STUDIES OF CYTOKININ METABOLISM

A thesis submitted for the degree of

Master of Science

in the Australian National University

by

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April, 1976

Statement

This thesis presents an account of research carried out by myself in the Department of Developmental Biology, Research School of Biological Sciences, The Australian National University, Canberra. Assistance and collaboration are recorded under acknowledgements and in the text.

C.W. Parker

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the interest, guidance and encouragement of my supervisor, Dr. D.S. Letham, throughout the experimental work and writing of this thesis. I am also indebted to him for permission to quote some of his hitherto unpublished results.

I am grateful to Professor D.J. Carr for permission to undertake this thesis and for the use of facilities in the Department of Developmental Biology. I also thank him for his encouragement, particularly during the latter stages of preparation of the thesis.

Thanks are also expressed to Dr. J.K. MacLeod, Dr. R.E. Summons and their associates in the Research School of Chemistry for providing the mass spectra presented herein. The assistance of Margaret Wilson with cotyledon picking for many of the bioassays was greatly appreciated.

Appreciation is also expressed for the excellent service provided by the photography/illustrating group in the School.

Working conditions were considerably enhanced by R.M. O'Connor, C.R. McArthur, Dr. I.J. Ryrie and numerous other members of the School.

Warm thanks are also expressed to Gill Mayoh for her patience, good humour and resourcefulness throughout the typing of this thesis.

TABLE OF CONTENTS

The publications listed below are based on work presented in this thesis.

1. PARKER, C.W. and D.S. LETHAM, 1974. Planta 115:337.
2. *PARKER, C.W., D.S. LETHAM, M.M. WILSON, I.D. JENKINS, J.K. MacLEOD and R.E. SUMMONS, 1975. Annals of Botany 39:375.
3. *MacLEOD, J.K., R.E. SUMMONS, C.W. PARKER and D.S. LETHAM, 1975. J. of Chem. Soc., Chemical Communications p. 809.
4. COWLEY, D.E., I.D. JENKINS, J.K. MacLEOD, R.E. SUMMONS, D.S. LETHAM, M.M. WILSON and C.W. PARKER, 1975. Tetrahedron Letters, p. 1015.

*These are preliminary publications from the work in chapter 3.

1.1. INTRODUCTION

Cytokinins are best defined as substances which stimulate cell division and growth of certain tissue cultures such as tobacco pith and soybean when grown on defined media containing an auxin. Together with auxins and gibberellins, the cytokinins play important roles in the regulation of cell division, cell enlargement, differentiation and organogenesis in developing plants. The first experimental evidence for the existence of cell division factors in plants was provided by Haberlandt (see review by Fox, 1969). In 1913 he showed that phloem diffusates induced cell division when applied to potato parenchyma and later in 1921 that cell division following wounding could be prevented by rinsing the wounded surface and restored by application of crushed tissue to the wound surface in several plant tissues. In subsequent years the discovery and identification of auxin and an examination of its physiological properties attracted great attention and the growth factors detected by Haberlandt were largely overlooked. However, the work of Skoog and co-workers and particularly the report published by Jablonski and Skoog (1954) clearly established that auxin alone could not induce cell division. They showed that cell division in tobacco pith tissues occurred only when vascular strands were attached, or, in the case of severed pith tissues, when they were placed in contact with vascular tissues. Severed pith tissues in the presence of auxin only, grew by cell enlargement and showed no cell division. This work

was the first real indication of the existence of a cell division factor *per se* which was required in addition to auxin.

Subsequent attempts to purify this factor from tobacco vascular tissue were unsuccessful. However, the search for the factor was continued and resulted in the detection of activity in such diverse sources as coconut milk, malt extract, yeast extract and autoclaved herring sperm DNA. The herring sperm DNA factor was purified and identified by Miller and associates (Miller *et al.*, 1955). This first cytokinin was shown to be 6-furfurylaminopurine and was believed to have been formed by the molecular rearrangement of deoxyadenosine freed from the DNA during autoclaving (Hall and de Ropp, 1955). The trivial name, kinetin, was adopted for this compound.

The discovery of kinetin stimulated much new research. Its biochemical and physiological effects were studied extensively; numerous analogues were synthesized, some of which proved to be highly active; kinetin-like activity was demonstrated in a variety of plant extracts. It was some years, however, before the first naturally occurring cytokinin, zeatin, was isolated. Zeatin and related compounds have been shown to occur widely in plants. The occurrence, identity, biosynthesis, translocation and metabolism of cytokinins will be discussed in this introductory chapter. Since the experimental part of this thesis is not concerned with the mechanism of cytokinin action or with effects of cytokinins on growth, these

aspects are not covered in the present chapter.

1.2. OCCURRENCE

Cytokinins are known to occur in nature in the free form (i.e. extractable by solvents such as ethanol) and also as bases in tRNA. Free cytokinins have been detected in diverse genera of higher plants. Higher plant tissueswhich are particularly rich sources of free cytokinins are those undergoing active cell division, notably root tips, developing fruit and germinating seed. They are also produced by certain bacteria and fungi and are secreted by the labial glands of a species of small caterpillar. However this discussion is limited to the occurrence of cytokinins in higher plants and in tRNA.

1.2.1. Occurrence in germinating seed

The occurrence of free cytokinins in germinating seeds has received considerable attention. Extracts prepared from germinating seeds of diverse genera have been shown to contain cytokinins (see review by Letham, 1967). In some species, increases in cytokinin levels in response to environmental stimuli have been observed prior to germination.

Several studies are concerned with the effect of stratification on the level of cytokinin activity in seeds. Sugar maple seed kept at 20°C for various periods does not germinate when transferred to suitable germination conditions and no cytokinin activity could be detected in them (van Staden et al., 1972). However, seed subjected to chilling was able to germinate and cytokinin activity was

extractable from the stratified seed. The highest level of cytokinin activity was found in seed chilled for 20 days (van Staden et al., 1972). Surprisingly, no cytokinin was detected in the germinated seed. Stratification of dormant seed of Protea compacta (embryo dormant) and of Leucadendron daphnoides (coat-imposed dormancy) resulted in an increased level of butanol extractable cytokinins (Brown and van Staden, 1973). In the former seed, these cytokinins appear to be either synthesised or released from a bound form; in the latter they are probably derived from cytokinins which are not extractable by butanol (Brown and van Staden, 1973). In contrast, no marked changes were detected in cytokinin level during stratification of sycamore seeds; only a gradual decline in level was observed (Webb et al., 1973).

In seeds of Rumex obtusifolius and Lactuca sativa, cytokinin levels rise rapidly in response to red light prior to germination (van Staden and Wareing, 1972; van Staden, 1973). Germination of Spergula arvensis seed is promoted by treatment with ethylene and the process appears to be under phytochrome control (van Staden et al., 1973). The breaking of dormancy in this seed by light and ethylene is accompanied by a marked increase in the level of cytokinin activity. This response occurs prior to germination (van Staden et al., 1973). When dormant seeds of lettuce are allowed to imbibe GA_3 solution in darkness, germination is induced and the level of butanol extractable cytokinins increases (van Staden, 1973).

Dormancy of Leucadendron daphnoides seed is apparently

due to the restricting effect of the seed coat on oxygen diffusion to the embryo. High oxygen tensions markedly promote germination and cause a large increase in butanol extractable cytokinins before germination is detectable (van Staden and Brown, 1973; Brown and van Staden, 1975).

1.2.2. Occurrence in fruit

Developing fruits of many species have proved to be rich sources of cytokinins. Active extracts have been obtained from apple, quince, pear, plum, peach and tomato fruitlets (see review by Letham, 1967). Bottomley et al. (1963) showed that the distribution of cytokinin activity within tomato and apple fruitlets paralleled the intensity of cell division in the component tissues. The cytokinin activities of apple and plum fruitlets have been studied in relation to the cell division periods of these fruits (Letham, 1963). With apple extracts, activity was greatest during the period of intense cell division following fertilization, and declined just before the cessation of cell division, but in plum fruitlets, highest activity occurred at the onset of active cell division. Letham and Williams (1969) compared the cytokinin activities of extracts of organs developed from the apple fruit bud. The activity of apple fruitlet extracts was slightly greater than that of pedicel extracts, and considerably greater than that of the extracts of other organs. They also found that the extract of the developing seed was very much more active than all other extracts and suggested that it may be a biosynthetic site. In an investigation

of the cytokinin content of pea seeds during development, Burrows and Carr (1970) observed that the two principal maxima in cytokinin content were coincident with the two maxima in the growth rate of the whole seed and the embryo.

In tomato fruits, the level of butanol-extractable cytokinins was greatest during the first two weeks following anthesis, that is, just before and during the early stages of rapid cell division (Abdel-Rahman et al., 1975). Later it declined very rapidly and then remained very low. The level of nucleotide cytokinins in tomato fruits was also very high during the first two weeks of development. It then declined rapidly but later increased (Abdel-Rahman et al., 1975). The growth and ripening of tomato fruits with different levels of endogenous cytokinins has been studied (Varga and Bruinsma, 1974). Parthenocarpic fruits showed retarded development and a very low cytokinin content. With seeded fruits, reduction of foliage increased the cytokinin level.

Cytokinin levels in avocado seed have been studied during fruit development (Blumenfeld and Gazit, 1970). The cytokinin content of the endosperm is very high throughout the period this tissue exists and exceeds that of the seed coat and embryo. The level in these tissues is greatest two to three weeks after fruit set and then decreases markedly. Cytokinin activity was detected in methanol extracts of avocado fruit mesocarp only after acid hydrolysis (Gazit and Blumenfeld, 1970). These authors suggest the extracts contain a bound cytokinin which is liberated

by acid. The level of this activity was high in very immature fruit but declined markedly as the fruit developed.

The peak of cytokinin activity in cotton fruits occurs from the fourth to the ninth days after anthesis; however 15 days after anthesis, cytokinin activity was not detected (Sandstedt, 1971). The cytokinin level in pumpkin seeds (Cucurbita pepo) reaches its maximum about 11 days after pollination and then decreases rapidly as the seeds develop further (Gupta and Maheshwari, 1970).

In contrast with the above observations, the maximum cytokinin activity in developing seeds of Gingko biloba occurs prior to fertilization when the gametophyte cells are dividing actively (Banerjee, 1968). The activity in the gametophyte tissue greatly exceeds that in the nucellus-integument tissues.

Several studies of the levels of cytokinin activity in developing seeds of monocotyledons have been reported. Cytokinin activity in sweet corn (Zea mays) kernels is maximal about 11 days after pollination (Miller, 1967). Levels of activity are much higher in rice ears at the heading and milk stages than at the fully ripe stage (Oritani and Yoshida, 1971). The cytokinin level in barley caryopses decreases very rapidly after pollination; differences in final grain size were positively correlated with differences in cytokinin content (Michael and Seiber-Kelbitsch, 1972).

1.2.3. Occurrence in xylem and phloem sap

The early work of Kulaeva (1962), Kende (1964, 1965),

Loeffler and van Overbeek (1964) and Nitsch and Nitsch (1965) established the presence of cytokinins in xylem sap and since then these hormones have been detected in the bleeding sap of various genera (see e.g. Carr and Reid, 1968). After decapitation of sunflower plants, the content of cytokinins in the sap was found to remain constant over 4 days (Kende, 1965); however this result was not in accord with the results of Carr and Reid (1968) who observed a steady decline in cytokinin content. Zeatin riboside appears to be the principal cytokinin in the xylem sap of a number of woody plant species (Hewett and Wareing, 1973a; Horgan et al., 1973).

Luckwill and Whyte (1968) detected increased cytokinin levels in apple sap prior to the occurrence of full bloom. Cytokinins were not present at detectable levels in the xylem sap of Populus x robusta (poplar) in mid-winter; however, the content increased rapidly in late winter and reached a maximum two weeks prior to bud burst (Hewett and Wareing, 1973a). In willow sap, cytokinin levels increased abruptly at about the time of flower-bud burst and this event took place at least six weeks after the level of total solids had increased. A second peak of cytokinin activity approximately coincided with leaf bud burst, afterwhich the cytokinin level declined rapidly and remained very low for the remainder of the season (Hewett and Wareing, 1974). The concentration of cytokinins in xylem sap of Perilla frutescens rose markedly when the plants were exposed to conditions (short days) causing

floral induction. Absolute amounts of cytokinins exported from the root exhibited a similar trend (Beever and Woolhouse, 1973). The increased cytokinin export from the roots associated with flowering in Perilla probably results in a higher cytokinin level in the leaves since leaf senescence is retarded in florally-induced plants (Beever and Woolhouse, 1974). The cytokinin content of the root exudate of sunflower plants increases during the exponential growth phase of the plants but declines very markedly at the end of the growth period (Sitton et al., 1967). Decreased cytokinin supply from the roots is probably one factor which contributes to shoot senescence.

The occurrence of cytokinins in phloem sap has now been established. Honeydew produced by aphids feeding on both flowering and vegetative Xanthium plants contains a cytokinin which cochromatographs with zeatin riboside (Phillips and Cleland, 1972). Aphids feed primarily on phloem sap and the honeydew produced is regarded as qualitatively similar to phloem sap. No cytokinin activity was detectable in honeydew obtained from aphids feeding on a chemically defined diet. Honeydew from aphids feeding on flowering plants contains a much higher level of cytokinin than honeydew from vegetative plants (Phillips and Cleland, 1972). In the above study, the unlikely possibility that aphids synthesize cytokinin from precursors present in the phloem sap was not eliminated. However, cytokinins have since been detected in phloem sap obtained directly from plants. Cytokinin and also auxin and

gibberellin were detected in phloem exudate of Ricinus communis plants (Hall and Baker, 1972). A cytokinin, probably zeatin riboside-5'-phosphate, was detected in the phloem sap of the inflorescence stalk of Yucca flaccida (Vonk, 1974).

1.2.4. Occurrence in roots and tubers

The widespread occurrence of cytokinin activity in the xylem sap of plants suggested that the roots produce or store cytokinins which are supplied to the aerial parts of plants. Weiss and Vaadia (1965) found that extracts of sunflower root apices were rich in cytokinin activity, but that extracts of older root tissues were inactive. In a similar investigation, Short and Torrey (1972) examined the occurrence of cytokinin activity in serial segments of young seedling roots of pea. They found that the terminal 0-1.0 mm of root tip contained forty times more free cytokinin on a fresh weight or per cell basis than the next 1.0-5.0 mm root segment. Acid hydrolysates of the tRNA from the root tips also showed cytokinin activity, but there was twenty-seven times as much free cytokinin as there was bound in tRNA.

Stimulation of vascular cambial activity in cultured radish roots requires exogenous auxin and cytokinin (see references in Radin and Loomis, 1971). These workers studied changes in cytokinin levels in radish roots during maturation and assessed whether such changes could regulate cambial activity in radish roots in vivo. The increased levels of two cytokinins (possibly zeatin and zeatin

riboside-5'-phosphate) were correlated with initiation of cambial activity. These two cytokinins both induced cambial activity when supplied to roots cultured *in vitro* and are evenly distributed between the xylem and the outer regions of the roots. In contrast an unknown cytokinin, which does not appear to be associated with cambial activity, is localized in the xylem (Radin and Loomis, 1971). In the roots of rice plants the cytokinin level was found to decline markedly at flowering (Oritani and Yoshida, 1971).

The cytokinin level rises in potato tuber tissue near the end of dormancy. The outer regions of the tubers and particularly the regions around the eyes are sites of cytokinin accumulation (Engelbrecht and Bielinska-Czarnecka, 1972). Grating and cutting potato tuber tissue causes an increase in butanol-soluble cytokinins and these are probably associated with the stimulation of cell division caused by wounding (Conrad and Kohn, 1975). Budding of sweet potato tubers (*Ipomoea batatas*) is accompanied by a marked rise in the cytokinin level in the tubers (Oritani and Yoshida, 1971).

1.2.5. Occurrence in buds and leaves

Detailed studies of cytokinin levels in developing buds and leaves are confined to species of *Populus* and *Acer*. Appreciable levels of cytokinin activity were detected in resting maple buds (*Acer platanoides*) by Engelbrecht (1971). However, cytokinins could not be detected in dormant *Populus x robusta* buds in midwinter (December), but the cytokinin level increased markedly

in late February and early March and reached a maximum about the time of natural bud burst (Hewett and Wareing, 1973a). As buds expanded, a transient decrease in cytokinin level occurred. Excised shoots of poplar were also forced to bud burst at 20°C under long days and the cytokinin levels in the buds were assessed (Hewett and Wareing, 1973a). Maximum cytokinin levels were detected about the time of bud burst. The cytokinin level of sycamore (Acer pseudoplantanoides) buds was at a maximum just prior to bud burst (Hewett and Wareing, 1974).

The occurrence of cytokinins in leaves of Populus species and in leaves of monocotyledons is discussed in the introductions to chapters 4 and 2 respectively.

In Xanthium strumarium plants, cytokinins present in young, actively growing leaves and in buds are mainly in the form of bases and/or ribosides, while in mature leaves a high proportion of the cytokinin activity is probably due to nucleotides (van Staden and Wareing, 1972a). The cytokinin level in rice leaves declined as the leaves matured (Oritani and Yoshida, 1971).

While the level of butanol-extractable cytokinins in proximal portions of Streptocarpus molweniensis leaves changes little during summer and autumn, the level in distal portions changes markedly (van Staden, 1973). The cytokinin level in the blade of bean leaf cuttings increases very markedly soon after the petioles develop roots (Engelbrecht, 1972).

Changes in cytokinin levels in rose petals have been assessed. The level increases as the flower begins to open and then decreases to a low level (Mayak et al., 1972).

The cytokinin content of petals of a short-lived variety was less than that of a long-lived variety (Mayak and Halevy, 1970). These and other observations implicate cytokinins in the regulation of petal senescence.

1.2.6. Occurrence in tRNA

The cytokinins are unique among the phytohormones because of their occurrence in the bound form in tRNA. Cytokinins have now been found in tRNA preparations from a wide spectrum of organisms ranging from mycoplasma to man (see review: Skoog and Armstrong, 1970). In plants soluble RNA (sRNA) from corn seedlings, immature corn kernels, wheat germ, frozen spinach, pea shoots and roots and tobacco callus tissue has yielded cytokinins. The discovery of the exact location of the cytokinin moiety in tRNA molecules resulted from the pioneering work of Zachau et al. (1966). They sequenced two species of tRNA from brewer's yeast and established that the cytokinin 6-(3-methylbut-2-enylamino)purine occurred only once in the polynucleotide, adjacent to the 3' end of the anticodon in both species. This observation seems to apply to all cytokinin containing tRNA species that have been sequenced. All tRNA species known to contain a cytokinin correspond to codons with the initial letter U (Skoog and Armstrong, 1970; Skoog, 1973). However, cytokinin activity has not been found in all tRNA species which translate codons with the initial letter U or even consistently in analogous tRNA species from different organisms (Skoog and Armstrong, 1970). Skoog and Armstrong have suggested the possibility

that all tRNA species recognizing codons beginning with U contain either a cytokinin or a cytokinin derivative. They believe that secondary modifications to the cytokinins might render them inactive in growth assays or labile in the usual preparative methods.

Initially the discovery of cytokinins in tRNA was believed to be the key to the elucidation of their mechanism of action as growth hormones. They definitely play an important role in tRNA function probably by promoting binding to the mRNA-ribosome complex, and possibly by modifying codon recognition (see review by Letham, 1973). However it remains uncertain if their occurrence in tRNA is related to their mechanism of action.

1.3. IDENTITY

The occurrence of cytokinins in nature has been widely reported, but chemical identification has been less common. Naturally occurring cytokinins that have been isolated in a state of complete purity and unequivocally identified are listed in Table 1.1 (free cytokinins) and Table 1.2 (cytokinins in tRNA). The structural formulae of these cytokinins are presented in Fig. 1.1. In 1963, the first cytokinin to be isolated in a state of purity was reported (Letham, 1963), 1.0 mg being obtained crystalline from about 60 kg of sweet corn (*Zea mays*) kernels. This compound, termed zeatin, was identified as 6-(4-hydroxy-3-methylbut-*trans*-2-enyl)aminopurine (I). Soon afterwards Letham identified zeatin riboside (II) and zeatin riboside 5'-phosphate (III) from the same source (Letham,

Table 1.1.

Free naturally occurring cytokinins of high activity and known structure and the sources from which isolation in a state of purity was achieved

	Compound	Source
I	6-(4-Hydroxy-3-methylbut-*trans*-2-enylamino)purine (zeatin)	*Zea mays* kernels (immature seed) (Letham, 1966a); *Rhizopogon roseolus* culture filtrates (Miller, 1967); cotton ovules (Shindy and Smith, 1975); unidentified ectendotrophic species of mycorrhizal fungus (Crafts and Miller, 1974)
II	6-(4-Hydroxy-3-methylbut-*trans*-2-enylamino)-9-β-D-ribofuranosylpurine (zeatin riboside)	*Zea mays* kernels (Letham, 1968; 1973); *Rhizopogon roseolus* (Miller, 1967); cotton ovules (Shindy and Smith, 1975); unidentified ectendotrophic species of mycorrhizal fungus (Crafts and Miller, 1974); chicory root (Bui-Dang-Ha and Nitsch, 1970); coconut milk (Letham, 1968; 1974); sycamore sap (Horgan *et al.*, 1973); *Vinca rosea* crown gall tumor tissue (Miller, 1974, 1975a)
III	Zeatin riboside 5'-monophosphate	*Zea mays* kernels (Letham, 1968, 1973)
IV	6-(3-Methylbut-2-enlyamino)purine (isopentenyladenine)	*Corynebacterium fascians* (Helgeson and Leonard, 1966; Rathbone and Hall, 1972); sea water (Pedersen, 1973); cotton ovules (Shindy and Smith, 1975)
V	6-(3-Methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (isopentenyladenosine)	Tobacco tissue, autonomous strain (Dyson and Hall, 1972); cotton ovules (Shindy and Smith, 1975)
VI	6-(4-Hydroxy-3-methylbutylamino)purine (dihydrozeatin)	Immature lupin seeds (Koshimizu *et al.*, 1967); cotton ovules (Shindy and Smith, 1975). The configuration of the asymmetric carbon has been established as S (Fujii and Ogawa, 1972)
VII	2-Hydroxy-6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-purine	*Zea mays* kernels (Letham, 1973)
VIII	6-(3,4-Dihydroxy-3-methylbutylamino)purine	*Zea mays* kernels (Letham, 1973)
IX	6-(2-Hydroxybenzylamino)-9-β-D-ribofuranosylpurine	*Populus* x *robusta* leaves (Horgan *et al.*, 1975)

Table 1.2.

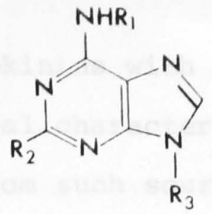
Cytokinins of known structure which occur in sRNA and the source of the sRNA

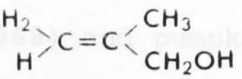
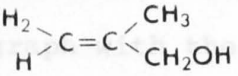
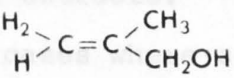
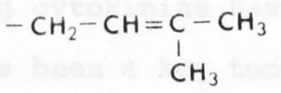
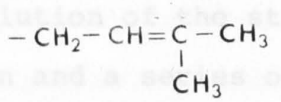
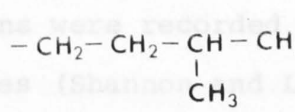
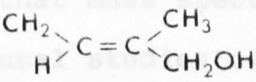
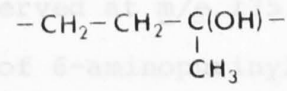
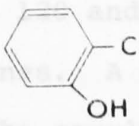
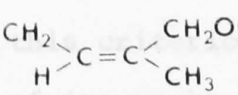
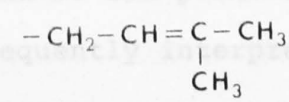
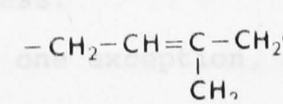
	Compound	Source
II	6-(4-Hydroxyl-3-methylbut-*trans*-2-enylamino)-9-β-D-ribofuranosylpurine (zeatin riboside)	sRNA from pea shoots[a] (Vreman *et al.*, 1972)
V	6-(3-Methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (isopentenyladenosine)	sRNA from plants, animal tissues, bacteria and yeast (see references in Gauss *et al.*, 1971 and Hall, 1973)
X	6-(4-Hydroxy-3-methylbut-*cis*-2-enylamino)-9-β-D-ribofuranosylpurine (*cis*-zeatin riboside)	sRNA of sweet corn (Hall *et al.*, 1967), wheat germ and tobacco callus tissue (Playtis and Leonard, 1971; Dyson and Hall, 1972), pea shoots[a] (Vreman *et al.*, 1972), pea roots (Babcock and Morris, 1970)
XI	6-(3-Methylbut-2-enylamino)-2-methylthio-9-β-D-ribofuranosylpurine	sRNA of wheat germ (Burrows *et al.*, 1970), pea shoots (Vreman *et al.*, 1972), bacteria (Gauss *et al.*, 1971; Hall, 1973)
XII	6-(4-Hydroxy-3-methylbut-2-enylamino)-2-methylthio-9-β-D-ribofuranosylpurine	sRNA of wheat germ (Burrows *et al.*, 1970), tobacco callus (Burrows *et al.*, 1971). pea shoots[b] (Vreman *et al.*, 1974) and *Pseudomonas aeruginosa* (Thimmappaya and Cherayil, 1974)

[a] Relative to *cis*-zeatin riboside, the amount of zeatin riboside is small

[b] XII from pea sRNA was shown to be a mixture of *cis* and *trans* isomers

Fig. 1.1. The structural formulae of naturally occurring cytokinins. Refer to Tables 1.1 and 1.2 for information regarding chemical name, source from which cytokinin was purified, and literature references.



	R_1	R_2	R_3
I		H	H
II		H	ribofuranosyl
III		H	ribofuranosyl 5′-phosphate
IV		H	H
V		H	ribofuranosyl
VI		H	H
VII		OH	H
VIII		H	H
IX		H	ribofuranosyl
X		H	ribofuranosyl
XI		CH_3S	ribofuranosyl
XII		CH_3S	ribofuranosyl

1966a, b, 1968). Cytokinins with the chromatographic and ultraviolet spectral characteristics of zeatin have since been purified from such sources as plum fruitlets (Letham, 1964, 1966c), immature sunflower fruits (Miller and Witham, 1964), sunflower leaves and root exudate (Klambt, 1968) and pumpkin seeds (Gupta and Maheshwari, 1970), while zeatin and zeatin riboside have been found to cochromatograph with the cytokinin activity in numerous other plant extracts.

In all cases where structural formulae of naturally occurring cytokinins have been elucidated, mass spectrometry has been a key tool. During the work which led to the resolution of the structure of zeatin, the mass spectra of zeatin and a series of 6-aminopurines including other cytokinins were recorded and rationalised in terms of their structures (Shannon and Letham, 1966). The results suggested that mass spectrometry would be a valuable asset for structural studies of these compounds. In particular, ions observed at m/e 135 and 108 were found to be characteristic of 6-aminopurinyl structures and those at m/e 149, 148, 121, 120 and 119 indicated the presence of 6-alkylaminopurines. A necessary criterion for mass spectrometry is that the sample be highly purified. Ignorance or neglect of this criterion results in spectra with a high background of ion peaks derived from the impurities present and consequently interpretation becomes difficult or meaningless.

With one exception, all naturally occurring cytokinins

of high activity can be regarded as derivatives of isopentenyladenine; the exception is 6-(o-hydroxybenzylamino)-9-β-D-ribofuranosylpurine, a compound isolated from mature leaves of poplar (Horgan et al., 1975). In this tissue it appears to occur free together with zeatin and zeatin riboside (Hewett and Wareing, 1973). Cytokinins which occur free in plants (Table 1.1) differ in some respects from those which are bound in tRNA (Table 1.2). cis-Zeatin occurs in sRNA, but unlike zeatin, it has not been found to occur in the free form. Cytokinin bases in sRNA may contain a 2-methylthio group; again these compounds, 6-(3-methylbut-2-enylamino)-2-methylthiopurine and 6-(4-hydroxy-3-methylbut-2-enylamino)-2-methylthio-9-β-D-ribofuranosylpurine, have not been detected in the free form in plants. The evolutionary significance of the occurrence of zeatin and cis-zeatin has been discussed (Kaminek, 1974).

All known cytokinins of high activity are 6-substituted purines. Such a statement prompts the question: what determines whether or not a particular derivative is active? The synthesis and testing of compounds for cytokinin activity, begun with the discovery of kinetin, has resulted in the discovery of many new cytokinins and has revealed some clear relationships between molecular structure and high cytokinin activity of purine derivatives (Skoog et al., 1967; Skoog and Armstrong, 1970). A detailed review of structure-activity relationships is beyond the scope of this chapter; however, the salient

points to emerge from these investigations are listed below.

1. There is a requirement for an intact purine moiety with a substituent of moderate size at the 6 position. Mono-substitution at any other position on the ring does not give cytokinin activity. The recent demonstration that certain 6-alkylpurines, which lack a 6-amino group, possess good cytokinin activity establishes that an intact adenine moiety is not essential for high activity; only an intact purine ring appears to be essential (Henderson et al., 1975).

2. Several properties of the side chain at position 6 are important for high cytokinin activity. In tests with 6-n-alkylaminopurines in the tobacco bioassay (Skoog et al., 1967), cytokinin activity was found to increase with increase in chain length to an optimum of five carbon atoms. Also, unsaturation of the side chain was found to contribute to the high activity of compound IV (Table 1.1) and zeatin. Removal of the double bond resulted in a ten-fold loss of activity in both cases. The location of the double bond did not appear critical although a slight loss of activity was observed when it was moved from the 2,3 to the 3,4 position. However, the 4 position is important in the location of the single hydroxyl group, a marked loss of activity occurring when it is moved to the 2 or 3 position in the side chain. Strongly polar substituents such as the carboxylic group caused a marked diminution of cytokinin activity. Some non-aliphatic side chains have also been found to confer a high degree of cytokinin activity on the adenine nucleus, the most notable being

the furfuryl group of kinetin and the benzyl group of the synthetic 6-benzylaminopurine. Overall, the evidence indicates that physical properties (dimensions, planarity, presence and distribution of polar groups), rather than the presence of a specific, chemically reactive group, are responsible for the degree of cytokinin activity conferred by the side chain on N^6-adenine derivatives.

3. Substitution by alkyl groups at the 1 and 3 positions of 6-(mono-substituted)aminopurines eliminates activity (Skoog et al., 1967). Substitutions at positions 2, 8 and 9 have a less drastic effect and naturally occurring 2-methylthio cytokinins are known (see Table 1.2, Fig. 1.1.).

Inevitably, during the search for other compounds with kinetin-like activity, non-purines were tested and some were claimed to be active. Shantz and Steward (1955) made the first such claim in the case of diphenylurea which had effects similar to cytokinin active N^6-adenine derivatives in some bioassays. The possibility that diphenylurea might serve as a side chain donor for reaction with adenine to form cytokinins has been suggested by Skoog. A comparison of almost 500 urea derivatives in several bioassays (Bruce and Zwar, 1966) to determine structure/activity relationships has established that some exhibit typical cytokinin activity although they are less effective than the adenine cytokinins. Other non-purines for which weak kinetin-like activity is claimed include the 8-aza analogues of kinetin, 6-benzylaminopurine and isopentenyl-

adenine and a diversity of other nitrogen heterocyclics.

1.4. SITES AND PATHWAYS OF CYTOKININ BIOSYNTHESIS

1.4.1. Sites of biosynthesis of free cytokinins

Numerous observations indicate that root tissues, and in particular root apices, are active sites of cytokinin production. Firstly, cytokinins are known to occur in xylem sap and their level in the sap is reduced markedly when the root system is subjected to stress or low temperatures (Atkin et al. 1973). Flooding is one form of stress which markedly reduces the cytokinin level in sap. During flooding of sunflower plants, Burrows and Carr (1969) observed a correlation between the metabolic activity of root apices and the cytokinin content of xylem sap. Secondly, the level of extractable cytokinins in root apices is very much greater than the level in more proximal regions of roots (Weiss and Vaadia, 1965; Short and Torrey, 1972a). Two cytokinins extracted from the root tips of sunflower plants are reported to be chromatographically identical to the two cytokinins of the xylem sap of this species (Kende and Sitton, 1967). Thirdly, these workers found the levels of the two cytokinins in sunflower root exudate did not decrease appreciably for four days after topping of the plants. Fourthly, root formation on petioles and treatment of the lamineae with cytokinins have very similar effects on the physiology of detached leaves (see references in review by Letham, 1967b). Fifthly, the level of cytokinins in the petioles of detached bean leaves increases greatly immediately after

thepetioles develop roots (Engelbrecht, 1972). Although cytokinins are not detectable in the leaf blades at or before this time, their level in the blades is subsequently much greater than that in the petioles plus roots. Sixthly, excision of the roots of Avena seedlings markedly reduces the growth of the coleoptiles; exogenous cytokinin completely restores the coleoptile growth rate in these derooted seedlings and stimulates auxin production by excised coleoptile tips (Jordan and Skoog, 1971). These results suggest that coleoptile growth and auxin production in the coleoptile tip depend on the supply of root-produced cytokinins. Hence, roots are not only organs of physical support and nutrient absorption, but are also responsible for supplying cytokinins and other hormones to the shoot. The cytokinins present in the xylem sap of plants are most likely synthesized in root apices.

There are many observations which indicate that roots are not the only site of cytokinin biosynthesis. Tomato, tobacco, pea and bean plants develop fruits even though the roots are removed at flowering and the formation of adventitious root primordia is prevented by periodic excision of the stem bases (Peterson and Fletcher, 1973). Tobacco fruits reach maturity on stems without roots and produce viable seed. The capacity for fruit and seed development under these conditions indicates that developing fruits and seeds are not completely dependent on roots for their supply of cytokinins and other hormones (Peterson and Fletcher, 1973). There are probably other sites of

cytokinin biosynthesis and from the evidence outlined below, one appears to be in the developing seed. The levels of cytokinin activity in developing apple (Letham and Williams, 1969) and avocado seed (Blumenfeld and Gazit, 1970; Gazit and Blumenfeld, 1970) are considerably greater than those of the receptacles and mesocarp, respectively. Extracts of developing apple seed are also much more active than extracts of all other parts of the developing apple fruit bud (Letham and Williams, 1969). Avocado cotyledon tissue derived from seed grows *in vitro* without exogenous cytokinin and actually produces cytokinins (Blumenfeld and Gazit, 1971). Parthenocarpic tomato fruits show retarded development and possess low levels of cytokinin compared with seeded fruits (Varga and Bruinsma, 1974). Excision of the developing seeds from apple fruitlet explants renders the growth of the explants in sterile culture more dependent on exogenous cytokinins (Letham and Bollard, 1961). Further evidence that cytokinins are produced by seed is provided by experiments in which pea pods excised 10 days after anthesis were grown in sterile culture on media lacking cytokinin (Hahn *et al.*, 1974). The pea seeds developed normally and reached full maturity; their cytokinin content rose dramatically during 18 days in culture (<0.01 to 3.3 isopentenyladenine equivalents per seed).

Considered overall, the above results indicate strongly that developing seeds are a site of active cytokinin biosynthesis. However at least two observations suggest the

root may also be a source of cytokinins for developing fruit tissue. The cytokinin content of tomato fruits increases if the number of fruits per cluster is reduced and this increase is greatly enhanced when the foliage is reduced by one third (Varga and Bruinsma, 1974); this result is consistent with competition between fruits and between fruits and leaves for cytokinins derived from elsewhere, presumably the root. During flower and fruit development in Perilla frutescens, the root system appears to provide the shoot with increased amounts of cytokinin (Beever and Woolhouse, 1973).

Germinating seed frequently yields extracts with appreciable cytokinin activity. Exogenous cytokinin can replace, at least in part, the embryonic axis in promoting the development of certain enzyme activities (e.g. amylase, protease) in cotyledons (Penner and Ashton, 1967a, b; Gepstain and Ilan, 1970; Locker and Ilan, 1975), and hence the axis appears to be a site of cytokinin biosynthesis in germinating seed. Unlike cytokinins, other hormones are unable to replace the stimulus of the embryonic axis. The regulation of degradation of storage carbohydrates and proteins in cotyledons by cytokinins produced in the axis appears to be a general phenomenon (Locker and Ilan, 1975).

In addition to the root tip, the developing seed and the embryonic axis, another possible site of cytokinin biosynthesis is the developing bud. Cytokinin levels increase in buds of excised poplar twigs, which lack roots, at about the time of bud burst (Hewett and Wareing,

1973a). Hence, cytokinins of developing poplar buds are not derived solely from the root and may be produced within the bud itself either by synthesis or by release from bound forms. From a comparison of the diffusible cytokinins in excised apical buds of Dahlia variabilis and of cytokinins in the xylem exudate of this species, Kannangara and Booth (1974) suggested that the apices are probable sites of cytokinin biosynthesis. However, the evidence for this is inconclusive. Experiments with the inhibitor hadacidin (inhibits adenylosuccinate synthetase) indicate that the cytokinin required for lateral pea bud development is synthesized locally in the bud itself (Kung-Woo Lee et al., 1974). The hadacidin inhibition does not appear to be transported to adjacent buds; the hadacidin inhibits bud development and presumably cytokinin biosynthesis when applied locally to lateral buds.

1.4.2. Pathways of biosynthesis of free cytokinins

There is considerable information concerning the biosynthetic origins of cytokinins in tRNA (see below) but relatively little is known about the biosynthesis of free cytokinins. tRNA itself, represents a potential source, but hydrolysis of the tRNA to mononucleotides would presumably be necessary to release the cytokinin. Although it is uncertain whether significant amounts of cytokinins are released from tRNA in living cells, this probably does occur in dying, autolysing cells of differentiating vascular tissues as suggested by Sheldrake (1973). However, some evidence points towards the existence of

alternative pathways for the biosynthesis of cytokinins. Firstly, Short and Torrey (1972) obtained 27 times more free cytokinin than could be obtained from tRNA in pea root tips. Therefore, unless a very rapid turnover of tRNA occurs in meristematic tissue, their results indicate that tRNA is not the principal source of free cytokinins (see Hall, 1973; cf. Klemen and Klambt, 1974). Secondly, lateral bud development in pea seedlings is dependent on cytokinin biosynthesis; studies with the inhibitor hadacidin indicate that this synthesis goes by a route similar to that for AMP and adenine (Kung-Woo Lee *et al.*, 1974). Thirdly, if free cytokinins are largely derived from the hydrolysis of tRNA, then the compounds X, XI and XII (see Table 1.2) which are present in tRNA would be expected to occur frequently as free cytokinins; none of these has been identified as a free naturally occurring cytokinin. Fourthly, there is good evidence that the fungus *Rhizopogon roseolus* synthesizes *N*-(purin-6-ylcarbamoyl)-threonine, a compound closely related to cytokinins, by a means independent of its synthesis in tRNA (Laloue and Hall, 1973). Fifthly, when callus cells derived from the sporogonium of the hybrid moss *Funaria hygrometrica* x *Physcomitrium piriforme* are supplied with labelled adenine, they produce a labelled cytokinin which has the same chromatographic properties as isopentenyladenine (IV of Table 1.1); weak cytokinin activity can be detected in hydrolysates of sRNA, but no radioactive cytokinin can be isolated from the sRNA of adenine-labelled callus cells (Beutelmann, 1973).

The enzyme reactions which yield free cytokinins independently of tRNA degradation are unknown. Transfer of an isopentenyl group from isopentenylpyrophosphate to adenine or adenosine, a reaction analogous to that which yields isopentenyladenosine in tRNA (see 1.4.3.), could be envisaged as a likely basic step in cytokinin biosynthesis. However, no evidence for its occurrence has been reported. Indeed, the inhibition of cytokinin biosynthesis by hadacidin, and the inability of adenine to alleviate it, suggests that a biosynthetic route involving inosine 5'-monophosphate may actually be involved (Kung-Woo Lee et al., 1974). Labelled adenine appears to be converted to cytokinins in plant tissues, but the percentage yield is very low (Miura and Miller, 1969; Beutelmann, 1973; Einset and Skoog, 1973). Regrettably, these studies provide no information regarding the biosynthetic pathways which are independent of tRNA.

Although the pathway to free isopentenyladenine is not clear, isopentenyladenine and isopentenyladenosine are precursors of zeatin riboside. Labelled isopentenyladenine is converted to zeatin riboside in very high yield by Rhizopogon roseolus (Miura and Miller, 1969), while isopentenyladenosine is trans-hydroxylated to yield zeatin riboside by both Rhizopogon roseolus and sweet corn endosperm (Miura and Hall, 1973).

1.4.3. Biosynthesis of cytokinins in tRNA

In the initial studies concerning biosynthesis of tRNA cytokinins, labelled mevalonate was supplied to certain

bacteria and to plant cells. When the mevalonate-requiring organisms, Lactobacillus acidophilus and L. plantarum, are grown in the presence of [2-^{14}C]mevalonate, the tRNA becomes labelled. All the radioactivity in the tRNA can be accounted for in the isopentenyl group of isopentenyladenosine (Fittler et al., 1968a; Peterkofsky, 1968). Similarly, when [2-^{14}C]mevalonate is supplied to tobacco pith tissue in culture, the tRNA is labelled and all of this radioactivity is in the isopentenyladenosine of the tRNA (Chen and Hall, 1969). However, when the same strain of tobacco pith tissue is cultured under different conditions and labelled with [2-^{14}C]mevalonate, Murai et al. (1975) found that approximately 40% of the label incorporated into tRNA was present in a ribonucleoside which was chromatographically identical to cis-zeatin riboside. Hence, like other isoprenoid constituents of cells, the isopentenyl group of tRNA cytokinins is derived from mevalonate. In vitro studies using cell free systems have established that the isopentenyl side chain is attached to the adenine moiety after completion of the tRNA polynucleotide chain. Treatment of tRNA with permanganate under mild conditions cleaves the isopentenyl side chains leaving adenine residues in place of isopentenyladenine. The resulting tRNA serves as a substrate for the attachment of the isopentenyl (i.e. 3-methyl-but-2-enyl) group in vitro. Crude enzyme preparations from yeast, rat liver and tobacco pith were found to catalyse the attachment of the isopentenyl group which was derived from labelled mevalonate supplied to the

in vitro system (Fittler *et al.*, 1968b; Chen and Hall, 1969). An enzyme has been purified from yeast which catalyses *in vitro* the transfer of the isopentenyl group from isopentenyl pyrophosphate to receptor adenosine residues of homologous, permanganate treated tRNA (Kline *et al.*, 1969). The enzyme is specific for the substrate, isopentenyl pyrophosphate (i.e. 3-methyl-but-2-enyl-pyrophosphate). There is good evidence that the isopentenyl groups are attached only to those adenosine residues in the tRNA from which the isopentenyl side chain has been removed (Kline *et al.*, 1969).

Similar transferase enzymes have been purified from *E. coli* (Bartz *et al.*, 1970; Rosenbaum and Gefter, 1972). These enzymes were shown to transfer the isopentenyl (3-methyl-but-2-enyl) group to naturally occurring species of tRNA which are deficient in this substituent. The group is most likely transferred to the adenosine adjacent to the 3' end of the anticodon (Rosenbaum and Gefter, 1972).

The enzymic synthesis of N-(purin-6-ylcarbamoyl) threonine (a base closely related to cytokinins in structure) in tRNA has also been investigated. An enzyme has been purified from *E. coli* which can synthesize this base in tRNA when supplied with L-threonine, bicarbonate, ATP, Mg^{2+} and tRNA deficient in the base (Elkins and Keller, 1974).

1.5. TRANSLOCATION

In early studies, little evidence was obtained for the movement of exogenous cytokinins away from the site of application. Thus, when kinetin was applied to the surface of leaves (Mothes, 1964) and lateral buds (Sachs and Thimann, 1964) its effects were extremely localized. In the latter case, a stimulation of lateral bud growth was observed only when kinetin was placed directly on the bud. Application to the stem or trifid bract, even within 2 mm of the bud tip, was without effect. These experiments indicated that cytokinins were immobile. Early evidence to the contrary was first derived from experiments involving the application of cytokinin to the cut ends of stems and petioles. Osborne and co-workers obtained evidence for the movement of BAP with predominantly basipetal polarity in petiole segments of Phaseolus vulgaris and observed that this movement was enhanced by the auxin indole acetic acid (Osborne and Black, 1964; Black and Osborne, 1965; Osborne and McCready, 1965). Lagerstedt and Langston (1966) confirmed the results of Osborne and Black (1964), although they did find that the polar movement of kinetin was dependent upon the age of the tissue and the kinetin concentration as well as upon the concentration of IAA. Other instances of basipetal cytokinin translocation have also been reported. Seth et al. (1966) applied ^{14}C-kinetin to the stumps of decapitated bean plants and found that simultaneous application of indole acetic acid (IAA) greatly increased basipetal movement of the kinetin.

Kinetin also moved with basipetal polarity when applied to segments of Zea mays roots, whether or not the root apex was present (El Saidi, 1971). Polar transport of labelled kinetin also occurred in segments of petioles, hypocotyls and roots of radish. Polarity was basipetal in petioles and hypocotyls, but acropetal in roots (Radin and Loomis, 1974). Piéniazek (1964) observed bud break on apple seedlings below the point of application of 6-benzylaminopurine (BAP).

However, basipetal movement of cytokinins does not appear to be a universal phenomenon in plants. Fox and Weis (1964) failed to find evidence for polar transport in petioles of Phaseolus vulgaris, coleoptiles of Avena sativa, epicotyls of Pisum sativum, or petioles of Coleus blumei, either in the presence or absence of IAA. Similarly, polar movement could not be detected in petioles of Xanthium pennsylvanicum (Osborne and McCready, 1965), stems or petioles of Gossypium hirsutum (Lagerstedt and Langston, 1966), stem sections of Lens (Pilet et al., 1967), mesocotyl and coleoptile segments of Zea mays (El Saidi, 1972), and sections of Cleome hassleriana stamen filaments, gynophores and pedicels (Koevenig, 1973).

The reported basipetal transport is of doubtful significance and has not been shown to occur under physiological conditions. The concentrations of cytokinins used in experiments in which polar transport has been observed (e.g. Black and Osborne, 1965) are approximately 400 times greater than optimum levels for callus growth, and the

possibility of artifacts being induced by such unphysiological levels cannot be excluded. Furthermore, the auxin IAA is known to influence cytokinin translocation in plants and abnormal auxin levels and hormone balance may well exist in the experiments discussed above.

Using autoradiography, Lagerstedt and Langston (1967) confirmed that kinetin was usually immobile when applied to the laminae of detached leaves except when applied directly over a vein, in which case it moved freely in the vascular system to the area distal to the point of application, suggesting entry into the transpiration stream. In experiments using intact or nearly intact plants, the labelled kinetin was transported basipetally in the main vein of the treated leaf, but acropetally in the stem of the plant (Lagerstedt and Langston, 1967). However, this acropetal movement may be simply a consequence of entry into the transpiration stream. When labelled BAP was applied to the stems of intact seedlings in a superficial paste (Pilet, 1968) or by injection (Guern et al., 1968), the radioactivity moved acropetally, and in the latter case a stimulation of growth was observed in the axillary buds above the sites of application. Lagerstedt and Langston (1967) showed that the roots of intact tobacco seedlings readily absorbed [8-^{14}C]kinetin and ^{14}C radioactivity was translocated throughout the plants. Translocated radioactive compounds were extracted from veinal tissue and shown to be active in a senescence cytokinin bioassay. Similar, but less decisive bioassay results were obtained from agar blocks inserted

into a notch cut into the main vein of tobacco leaves actively taking up labelled kinetin through their petioles. However, in neither experiment was the translocated cytokinin activity shown to be kinetin, itself. Gordon et al. (1974) supplied ^{3}H labelled zeatin to the roots of radish seedlings and showed that the hormone was translocated in the xylem sap as the riboside.

Considerable attention has been paid to cytokinin translocation in relation to lateral bud development. In studies using Cicer arietinum seedlings, Guern and Sadorge (1967) found that a developing lateral bud can guide the translocation of BAP. Thus, in decapitated seedlings with a developing axillary shoot, injected BAP moved preferentially into the axillary shoot whether the site of injection on the main stem was above or below the axillary shoot. Similar results were obtained by Morris and Winfield (1972) when they supplied [8-^{14}C]kinetin to either the roots or to the upper stem of dwarf pea seedlings 24 hours after removal of the apical bud. Decapitation resulted in the transport of significant amounts of ^{14}C to the axillary buds from either point of application. Pretreatment of the cut internode surface of decapitated plants with IAA (alone or in combination with unlabelled kinetin) inhibited the transport of label to the axillary buds, and instead the label moved as it did in intact plants, accumulating in the stem and apical region. Thus, exogenous auxin seems able to mimic the effect of the shoot apex on cytokinin transport. These experiments indicated the transport

of label from intact root to shoot but the chemical nature of the label was not established. Wooley and Wareing (1972a) have studied the relationships between the development of lateral buds and cytokinins in Solanum andigena. In this species the lateral buds have the potential to develop as stolons or as leafy orthotropic shoots. Lateral buds of decapitated plants tended to develop into stolons, rather than leafy shoots, if an IAA/GA_3 mixture was applied to the apical stump. When [^{14}C]BAP was supplied to such plants with roots excised, BAP and BAP riboside did accumulate to some extent in the tips of the induced stolons, but showed an increased accumulation there after removal of the IAA/GA_3 mixture from the apex. This increased cytokinin content may be associated with the resulting transformation of the stolon into a leafy shoot. In further experiments using rootless, decapitated two-node cuttings of Solanum andigena, Wooley and Wareing (1972b) observed that application of IAA to the upper cut surface suppresses the accumulation in buds of labelled BAP supplied at the base of the cutting. By contrast adenine does not stimulate lateral bud growth in decapitated cuttings, but radioactivity from this compound does appear to increase in the buds of cuttings treated with IAA. IAA did not appear to reduce BAP or BAP-riboside accumulation in lateral buds by diverting these cytokinins to the point of IAA application, but did promote the formation of an unknown metabolite of BAP which occurred in stem tissue (Wooley and Wareing, 1972b). Thus, auxin is apparently implicated in the

regulation of translocation and metabolism of cytokinins.

Evidence for radial and tangential transport of cytokinins in stems has also been reported. Bowen and Wareing (1969) have demonstrated a bidirectional interchange of [^{14}C]kinetin and [^{14}C]GA_3 (gibberellic acid) between the xylem and sieve tube sap of willow stems (Salix viminalis) and also detected considerable degradation of the kinetin in cells of the cortex and ray parenchyma. Wareing (1970) has also demonstrated tangential movement of kinetin in the same species. [^{14}C]kinetin applied to lateral abrasions on horizontal willow stem sections was shown to accumulate in buds on the upper surfaces. Similar experiments but involving the application of [^{14}C]IAA as well as [^{14}C]kinetin were carried out by Lepp and Peel (1971). They found that IAA moves preferentially into the lower half of a horizontal stem, kinetin into the upper half. As all the stem segments used in these experiments were debudded before use, it would seem that the asymmetric [^{14}C]kinetin distribution is not entirely dependent on their presence. In another experiment which examined the radial movement of labelled IAA and kinetin from the centre of the xylem to the bark, no difference in distribution between the upper and lower portions of the stem was detected (Lepp and Peel, 1971).

1.6. METABOLISM

The conversion of cytokinins into low-molecular-weight metabolites and also their incorporation into sRNA are discussed in this section. Since the experimental section of this thesis is concerned with low molecular weight metabolites of cytokinins, this field is reviewed in detail

only to the time at which the experimental work was begun. It is appropriate to discuss subsequent work in the discussion chapter.

1.6.1. Metabolism of 6-benzylaminopurine (BAP)

The first report on cytokinin metabolism was provided by McCalla et al. (1962). They supplied labelled BAP to leaf discs of Xanthium pennsylvanicum (cocklebur) and found that N_6-benzyladenosine was the major metabolite. Other metabolites formed were N_6-benzyladenylic, adenylic, guanylic and inosinic acids as well as small amounts of adenine and guanine. Label was also found in urea and an ureide and small amounts of labelled adenylic and guanylic acid but no N_6-benzyladenylic acid was recovered from leaf RNA. Conversion of BAP to its riboside has since been detected in other plant tissues (Guern et al., 1968; Dyson et al., 1972; Wooley and Wareing, 1972) and further evidence of BAP metabolism to its nucleotide has also been reported (Bezemer-Sybrandy and Veldstra, 1971; Dyson et al., 1972; Doree and Guern, 1973). McCalla et al. (1962) also examined BAP metabolism in leaves of Phaseolus vulgaris and reported the formation of a single major metabolite, which appeared different to those produced by Xanthium leaves. Fox et al. (1972) detected a very stable BAP metabolite in soybean tissue cultures which appeared to be active as a cytokinin (Dyson et al., 1972) and from preliminary observations suggested that the compound was a phosphate ester. However, this compound was subsequently tentatively identified as 6-benzylamino-7-gluco-

furanosylpurine (Deleuze et al., 1972); these workers have also provided evidence for the formation of this metabolite in potato tuber slices and intact seedlings of Lupinus luteus (Fox et al., 1973). A 6-benzylamino-7-glucosylpurine has been identified as a major metabolite in immature excised radish cotyledons and de-rooted seedlings (Wilson et al., 1974). An analogous metabolite of zeatin has also been identified in studies with radish (see 1.6.2.). Wilson et al. (1974) also identified a second major metabolite as 6-benzylamino-9-glucosylpurine; in addition several minor metabolites were detected one of which was 6-benzylaminopurine riboside while another ("metabolite C") appeared to possess high cytokinin activity. In contrast to the above reports of the rapid and extensive metabolism of BAP, Letham et al. (1972) found that 6-benzoylaminopurine (an amide of adenine) was quite stable when supplied to excised radish cotyledons and de-rooted seedlings. This compound was still the principal source of n-butanol extractable radioactivity present in both tissues (71% and 54%, respectively) many hours after the completion of uptake and no significant metabolism to BAP (<0.07%) or BAP riboside (<0.20%) was detected. In the same report, the high cytokinin activity of the 6-benzoylaminopurine was established in several bioassays.

1.6.2. Metabolism of zeatin

Compared with BAP metabolism studies, the literature contains relatively few reports concerning the metabolites formed when zeatin is supplied exogenously to plant

tissues. Sondheimer and Tzou (1971) supplied [^{14}C]zeatin to excised bean axes and on extracting the tissue 12 hours later detected radioactivity in zeatin, dihydrozeatin and their ribosides and 5'-ribotides. When supplied with [^{14}C]-zeatin, dormant and non-dormant embryos of ash (Fraxinus americana) have been shown to metabolize the hormone to zeatin riboside and the 5'-mono- and probably the di- and triphosphates (Tzou et al., 1973). However, dihydrozeatin or its derivatives were not detected in these tissues.

Parker and Letham (1973) supplied [^{3}H]zeatin through the transpiration stream to radish seedlings with roots excised. Formation of dihydrozeatin was not detected but numerous other metabolites were formed, including adenine, adenosine, AMP, zeatin riboside and zeatin riboside-5'-monophosphate. However, in labelled seedlings which had been left in water for 15 hours, an unknown compound (raphanatin) was the dominant metabolite and accounted for about 25% of the total radioactivity extracted. Raphanatin was characterized by mass and ultra violet spectra and has been identified as 7-glucosylzeatin. It is an unusual compound in the nature of the sugar attached to the purine ring and also in the site of the glycosidic linkage. It is an active and very stable metabolite which was located mainly in the cotyledon laminae and may be a bound or storage form of the hormone. In contrast, labelled nucleotides, the other major metabolites of zeatin, were largely confined to the hypocotyls and petioles. Zeatin riboside-5'-monophosphate was the dominant metabolite in hypocotyls

of de-rooted seedlings supplied with zeatin for 0.5-2 hours. The majority of radioactivity in the xylem sap was due to zeatin, but about 10% was present as zeatin riboside; nucleotides accounted for less than 10% of the radioactivity and labelled raphanatin was not detected. In similar studies, but using intact seedlings, Gordon et al. (1974) also identified raphanatin as the dominant metabolite of zeatin present in radish roots and cotyledon laminae. AMP and zeatin riboside 5'-monophosphate were the principal metabolites in hypocotyl extracts whereas zeatin riboside was the only significant source of radioactivity in the xylem sap.

1.6.3. Incorporation of cytokinins into sRNA

The possible incorporation of cytokinins into tRNA is a controversial issue, but one of considerable interest, since such incorporation has been suggested as a mechanism of cytokinin action. A number of workers (Hall, 1968; Richmond et al., 1970; Bezemer-Sybrandy and Veldstra, 1971; Elliott and Murray, 1972) have failed to detect incorporation of labelled cytokinins into sRNA. Kende and Tavares (1968) found that the 9-methyl cytokinin, 6-benzylamino-9-methylpurine, was not incorporated into the sRNA of soybean callus tissue. On the other hand, Fox and Chen (1967) reported the incorporation of BAP into sRNA, while Burrows et al. (1971) identified N_6-benzyladenosine in the hydrolysate of sRNA isolated from tobacco callus tissue grown on BAP. These reports of incorporation have been criticised on the basis of the purity of the sRNA prepara-

tions (Kende, 1971 and references therein; see also Hall, 1973) and the demonstrated difficulty of freeing sRNA from non-covalently bound BAP and its metabolites (Elliott and Murray, 1972). The question of cytokinin incorporation into sRNA has recently been re-examined critically by Walker et al. (1974). These workers grew cytokinin-dependent tobacco callus tissue in the presence of a mixture of [8-^{14}C]6-benzylaminopurine and [benzene-^{3}H]6-benzylaminopurine and compared the $^{3}H/^{14}C$ ratio of BAP recovered from labelled tRNA preparations with the corresponding ratio in the medium. By this means, with suitable controls, the incorporation of BAP into the tRNA was shown to involve the intact moiety. The level of incorporation was very low - one molecule per 10,000 tRNA molecules. It is possible this is due to incorporation into the -CCA "tail" of tRNA which undergoes turnover. Such incorporation is of doubtful significance. Any role incorporated cytokinin might have would appear to be other than as a replacement for endogenous cytokinins in their known functions as constituents of tRNA.

1.7. THE PURPOSE OF THE INVESTIGATIONS DETAILED IN THIS THESIS

Studies of metabolism are potentially very useful for at least two reasons. Firstly, these studies may provide information leading to the identification of the active form or forms of cytokinins and hence help to answer the question: are compounds such as zeatin and 6-benzylaminopurine active per se, or are certain metabolites their

real functional forms? Secondly, there is evidence that bound (or storage?) forms of cytokinins exist in plants (see e.g. Gazit and Blumenfeld, 1970; Borkowska and Borkowski, 1975; Borkowska and Rudnicki, 1975). Studies of metabolites formed from exogenously supplied cytokinins may provide information regarding the identity of these weakly active or inactive substances which yield highly active cytokinins on hydrolysis.

The experimental chapters of this thesis discuss the metabolism of zeatin in three selected plant species, namely Zea mays (chapter 2), Lupinus angustifolius (chapter 3) and Populus alba (chapter 4). All of these studies are concerned with the naturally occurring cytokinin, zeatin. However, the author also participated in an investigation of a metabolite of 6-benzylaminopurine formed in radish (Raphanus sativus) seedlings (metabolite C of Wilson et al., 1974). Since this study has already been published (Letham et al., 1975) and since the author contributed experimentally to only part of the work, namely the initial purification steps, the investigation is not presented as a chapter. Nevertheless, this publication is relevant to the discussion of the results presented in this thesis and hence a photocopy is attached as an appendix.

2.1. INTRODUCTION

2.1.1. In the first experimental chapter of this thesis a study of cytokinin metabolism in Zea mays is reported. As an introduction to this study, it is desirable to consider the identity, occurrence and regulatory role of cytokinins in the Gramineae. There is considerable information regarding the identity of cytokinins in Zea mays kernels (see Chapter 1). The kernels are a particularly rich source in which the cytokinin level is maximal at about 11 days after pollination (Miller, 1967). However, the identity of cytokinins in other parts of the Zea mays plant and in other graminaceous species has not been established. Also, there is only limited information regarding cytokinin levels in tissues of plants belonging to this family.

The cytokinin activity of the xylem sap of Zea mays has been shown to vary markedly with root temperature but was maximal at a temperature of 28°C. Xylem sap corresponding to a root temperature of 28°C contained three unidentified cytokinins (Atkin, 1973). The first demonstration of cytokinins in leaves of a monocotyledon was provided by Oritani and Yoshida (1971). These workers detected cytokinin activity in the leaves and roots of rice plants and found that the levels of cytokinins present in both these organs declined markedly with the onset of senescence and flowering. Three cytokinins were detected in root exudate of rice plants and chromatographic characterization suggested that these may be zeatin,

zeatin riboside and zeatin nucleotide (Yoshida et al., 1971). An unidentified cytokinin was detected in the roots of rice plants by Yoshida and Oritani (1972) and they presented limited evidence which suggested it may be a glucoside of zeatin; the identity of the sugar was not established unequivocally and evidence regarding the site of the glycosidic linkage was not presented. Cytokinin activity in barley caryopses declines very rapidly after pollination; differences in final grain size were positively correlated with differences in cytokinin content (Michael and Seiler-Kelbitsch, 1972). Weak cytokinin activity was also detected in the etiolated first leaves of barley prior to unrolling and this level did not increase following irradiation with red light (Carr et al., 1972). Van Onckelen and Verbeek (1972) have partially purified a cytokinin from 3-day-old barley seedlings. In wheat leaves, the longitudinal distribution of cytokinin activity varied considerably depending on leaf maturity; cytokinin activity was also detected in guttation drops from wheat leaf tips (Wheeler, 1973).

In the leaves of the Gramineae, exogenously supplied cytokinins evoke a number of marked physiological responses which are not caused by other known phytohormones at physiological concentrations. Cytokinins at very low concentrations retard the senescence of barley (Kende, 1965; Carr and Burrows, 1966), oat (Thimann and Sachs, 1966; Varga and Bruinsma, 1973) and wheat leaves (Rothwell and Wright, 1967). Cytokinin bioassays have

been based on these responses; the barley leaf assay is extremely sensitive and highly specific for cytokinins, auxins and gibberellic acid being completely inactive. Retardation of senescence of sweet corn leaves by cytokinins has also been reported (Letham, 1967). In senescing corn leaves, cytokinins markedly inhibit protein degradation and this may result in retardation of senescence (Tavares and Kende, 1970).

The control of stomatal aperture in graminaceous species is another physiological function which has been directly associated with the cytokinins. Cytokinins have been shown to stimulate stomatal opening in these species but not in other monocotyledons or in dicotyledons (Luke and Freeman, 1968, Cooper et al., 1972). In barley there is evidence of an interaction between kinetin and abscisic acid to control stomatal aperture (Cooper et al., 1972). This influence over stomatal aperture and hence transpiration has provided the basis for a further bioassay using excised oat leaves which are more sensitive than wheat or barley (Luke and Freeman, 1967); auxins and gibberellic acid were inactive in the assay (Luke and Freeman, 1968). Kinetin has been found to cause a rapid increase in the rate of carbon dioxide assimilation in barley leaves (Meidner, 1967). This response and the effect of cytokinins on stomata may be linked.

When applied to leaves, cytokinins have the ability to direct the transport of nutrients and metabolites; these effects are particularly pronounced in leaves of

graminaceous species. Thus phosphate, glycine and photosynthate moved from the tip of excised oat leaves to accumulate at the site of localised kinetin application near the base (Gunning and Barkley, 1963). Other plant hormones did not cause an accumulation of these metabolites. Basipetal transport of $^{32}PO_4$ towards the natural mobilization centre at the base of corn leaves was accelerated by the application of kinetin near the leaf base and the $^{32}PO_4$ accumulated in the treated area (Muller and Leopold, 1966). Kinetin treatment at the leaf tip can suppress the normal basipetal flow of $^{32}PO_4$ and when the leaf base is excised acropetal transport is induced by kinetin (Muller and Leopold, 1966). Dekhuijzen and Staples (1968) have used cytokinin directed transport in oat leaves as a qualitative bioassay for cytokinins.

From the above observations, it is evident that basic aspects of leaf physiology in graminaceous plants are markedly stimulated by cytokinins, but not by auxins or gibberellins. Hence in leaves of this family, the cytokinin level is probably low and limiting and changes in this level are probably of regulatory significance. Indeed hormonal regulation in intact leaves of the Gramineae may be largely achieved through changes in the cytokinin level caused either by altered rates of biosynthesis or by degradation and inactivation of cytokinins. Hence a study of cytokinin metabolism in a graminaceous plant appeared particularly worthwhile and is reported herein. The growth responses of barley plants and Avena seedlings to

exogenous cytokinins emphasise the importance of cytokinins in regulation of development of graminaceous plants (Jordan and Skoog, 1971; Ruckenbauer and Kirby, 1973).

2.2. EXPERIMENTAL

2.2.1. Uptake of [^{3}H]zeatin by Zea mays seedlings and cultured tissue; extraction of tissue

[G-^{3}H]Zeatin (194 mCi/mmole), prepared according to Letham and Young (1971), was obtained from Dr. D.S. Letham. The roots of 10-day-old, intact Zea mays seedlings were placed in [G-^{3}H]zeatin solution (8 μM) for 20 hours, after which time they were excised, washed with distilled water and blotted between filter papers before weighing. The excised roots were extracted by immersion in 80% methanol (12 ml/g of tissue) at 65°C. The mixture was held at this temperature for five minutes, rapidly cooled to room temperature, and finally homogenized. The extract was filtered and the clarified solution evaporated to dryness in vacuo at <40°C using a rotary film evaporator. The same method was employed for the other extractions mentioned below.

The roots were excised from other seedlings and the stem bases were placed in [G-^{3}H]zeatin solution (8 μM) for 8 hours and then in distilled water for 14 hours. At the end of this time the de-rooted seedlings were extracted with 80% methanol. In both of the above experiments, all seedlings were continuously exposed to a gentle air current under light from white fluorescent tubes (about 700 lux).

Undifferentiated tissue which was derived from Zea mays embryos was cultured on an agar medium (see Gresshoff and Doy, 1973). Some of this tissue was then gently shaken for 17 hours in the culture medium of Miller (1968) which had

been modified by the omission of agar, sucrose, auxin and kinetin and by the addition of [G-^{3}H]zeatin (8 μM). This tissue was then washed with distilled water and extracted with 80% methanol.

2.2.2. Preparation of sap from Zea mays seedlings

Sixteen Zea mays seedlings were grown for twelve days in sand and then deprived of water for 24 hours. They were then 'watered' with 100 ml of a 16 μM [G-^{3}H]zeatin solution. After 36 hours, the seedlings were decapitated just above the sand level. The exudate which appeared immediately after decapitation was collected by blotting with filter paper and discarded. This was done because this exudate may have contained a considerable amount of non-sap material. The subsequent exudate was collected by absorption into small wedges of filter paper which were immediately dropped into 1 ml of 50% methanol. The eluate was collected, evaporated to dryness and taken up in 500 μl of 50% methanol for chromatography.

At the end of 8 days, the decapitated seedlings had regenerated considerable amounts of leaf tissue. In preparation for the extraction of the root tissue of these seedlings, the supporting sand was washed repeatedly over 72 hours to remove labelled zeatin. The seedlings were then 'watered' regularly with Hoagland's solution over a period of 4 days after which the roots were excised from the shoots, washed free of sand and extracted.

2.2.3. Chromatographic methods

i) Materials. After evaporation, all extracts were

dissolved in 50% ethanol for chromatography. The papers used were Schleicher and Schull 598L for all routine chromatography, and Schleicher and Schull 2040B, which had been exhaustively washed in 20% redistilled ethanol, for the final purification before mass spectrometry of the unknown compound Y. Thin layer chromatography (TLC) was done on layers of Merck PF_{254} silica gel (40g slurried with 85 ml water) or Serva DEAE cellulose. When borate impregnated silica gel layers were required, they were prepared by slurrying the silica gel with 0.05 M sodium tetraborate. To prepare DEAE cellulose layers, a mixture of the cellulose (14g), Woelm green fluorescent indicator and water (72 mls) were blended at high speed in preparation for spreading. Layers for preparative TLC were 1 mm thick; all other layers were 0.25 mm. For column chromatography, the Whatman modified floc celluloses DE1 (diethylaminoethyl) and P1 (cellulose phosphate) were used in the HCO_3^- and NH_4^+ forms, respectively.

The solvents used for paper chromatography and TLC were (proportions are v/v):

A: butan-1-ol-14N ammonia-water (6:1:2, upper phase)

B: butan-1-ol-acetic acid-water (12:3:5)

C: butan-1-ol saturated with water

I: 2-ethoxyethanol-water (3:1) saturated with sodium tetraborate

J: ethanol-water (2:1) saturated with sodium tetraborate

K: 2-ethoxyethanol-water (3:1)

L: ethanol-water (2:1)

M: methyl ethyl ketone-water-acetic acid (16:4:1).

ii) Detection and elution of compounds. UV-absorbing zones on chromatograms were detected using a short-wavelength UV lamp. Zones from paper chromatograms required for rechromatography were cut out and exhaustively eluted with 0.2 N acetic acid. Zones from silica-gel preparative TLC were scraped off the plates and eluted twice with 80% methanol. Silica gel TLC zones for liquid scintillation counting were eluted with 0.2 N acetic acid whereas DEAE-cellulose TLC zones were eluted in 0.1 N HCl containing NaCl (0.4 M).

2.2.4. Characterization of chromatographic fractions

A considerable proportion of the radioactivity on paper chromatograms (solvent A) of plant extracts was often found at and near the origin, the known location of nucleotides. Hydrolysis by alkaline phosphatase (E. coli, Sigma type IIIS, 0.30 mg/ml, pH 9-10) for 3 hours at 35°C, followed by paper chromatography (solvent A), was used to characterize these nucleotides.

Confirmatory evidence regarding nucleotide identity was obtained by degradation to the corresponding base. An aqueous solution of the evaporated eluate (50 µl) containing sodium periodate (0.5 mg) was left at 35°C for 12 hours. Cyclohexylamine (10 µl) was then added and the mixture was left at 35°C for 3 hours before being chromatographed. Only nucleoside-5'-phosphates were degraded under these conditions. This method is an adaptation of the procedure of Yu and Zamecnik (1960) for degrading

nucleosides to bases.

2.2.5. Determination of radioactivity

Chromatogram zones for counting were eluted in scintillation vials either at room temperature overnight or at 50°C for 3 hours. The eluates were mixed with 10 volumes of scintillant (toluene-Triton X-100, 2:1 v/v, containing PPO, 2 g/l and dimethyl POPOP, 0.2 g/l) and counted in a Beckman LS-250 liquid scintillation spectrometer. Levels of background radioactivity were determined for each chromatogram from blank zones taken from the unlabelled margins of the paper or layer. Vials from chromatograms of low activity were counted several times and the mean count calculated for each zone.

2.2.6. Purification of root metabolite Y

The roots of Zea mays seedlings which had been in zeatin solution (50 μM) for 20 hours were excised from the plants, washed with water and extracted with 80% methanol. The residue obtained after evaporation of the extract was suspended in water (100 ml) and then centrifuged. The resulting supernatant was percolated through a column of DEAE-cellulose (400 ml, HCO_3^- form) which was then washed with water (2.5 l). The combined effluents were concentrated to 150 ml, adjusted to pH 3.0 with acetic acid, and percolated through a column of cellulose phosphate (180 ml, NH_4^+ form equilibrated to pH 3.0). The column was washed with water (500 ml) acidified to pH 3.0 with acetic acid, and then eluted with 0.3 N aqueous ammonia (1.3 l). The residue obtained by evaporation of the eluate was subjected

to two preparative TLC purifications on silica gel. ^{3}H-labelled metabolite Y eluted from the paper chromatogram depicted in Fig. 2.1B was cochromatographed to enable the desired zone to be located in each purification. Chromatography with solvent B yielded a Y-containing zone at R_f 0.44; this was eluted and the eluate was rechromatographed using solvent A. The resulting Y zone (R_f 0.20), which was visible under UV light, was eluted and chromatographed on exhaustively washed paper using solvent C (Chromatogram developed in an atmosphere of ammonia). The zone (R_f 0.43) containing Y was eluted with ethanol and rechromatographed on paper using the same solvent system to yield purified Y (35 µg approximately). This was eluted with ethanol for UV and mass spectra and chemical degradation.

2.2.7. Methods used in structural studies of Y

UV spectra were determined with a UNICAM SP.800 spectrophotometer. Mass spectra of underivatized compounds were obtained on an AEI MS-902 instrument operated at 70eV. Samples for mass spectrometry were dissolved in methanol and aliquots evaporated directly onto the solid sample probe.

Mass spectrometry indicated that metabolite Y was a zeatin-hexose conjugate and the nature of the hexose moiety was examined by chemical degradation. Y (7 µg, based on UV data) was dissolved in 100 µl of 0.1 N acetic acid and 12 mg of Zeokarb 225 (SRC 14, polystyrene sulphonic acid resin converted to H^+ form with 6 N HCl, washed thoroughly with water, heated to 100°C in water, washed again) were added.

The suspension was stirred for 15 min. and then heated at 120°C in an autoclave for 1 hour. The supernatant was spotted onto a silica gel thin layer plate and developed in solvent L. The plate was dried in a warm air current and then placed in an atmosphere of ammonia for a few min. to neutralize any remaining traces of acetic acid. Any residual ammonia in the layer was removed by standing the plate in a cold air current for 5 min. after which the plate was sprayed with a mixture of glucose oxidase, peroxidase and o-dianisidine (see Salton, 1960). A pronounced pink-brown spot was observed at the same R_f as authentic marker glucose (R_f 0.18).

2.3. RESULTS

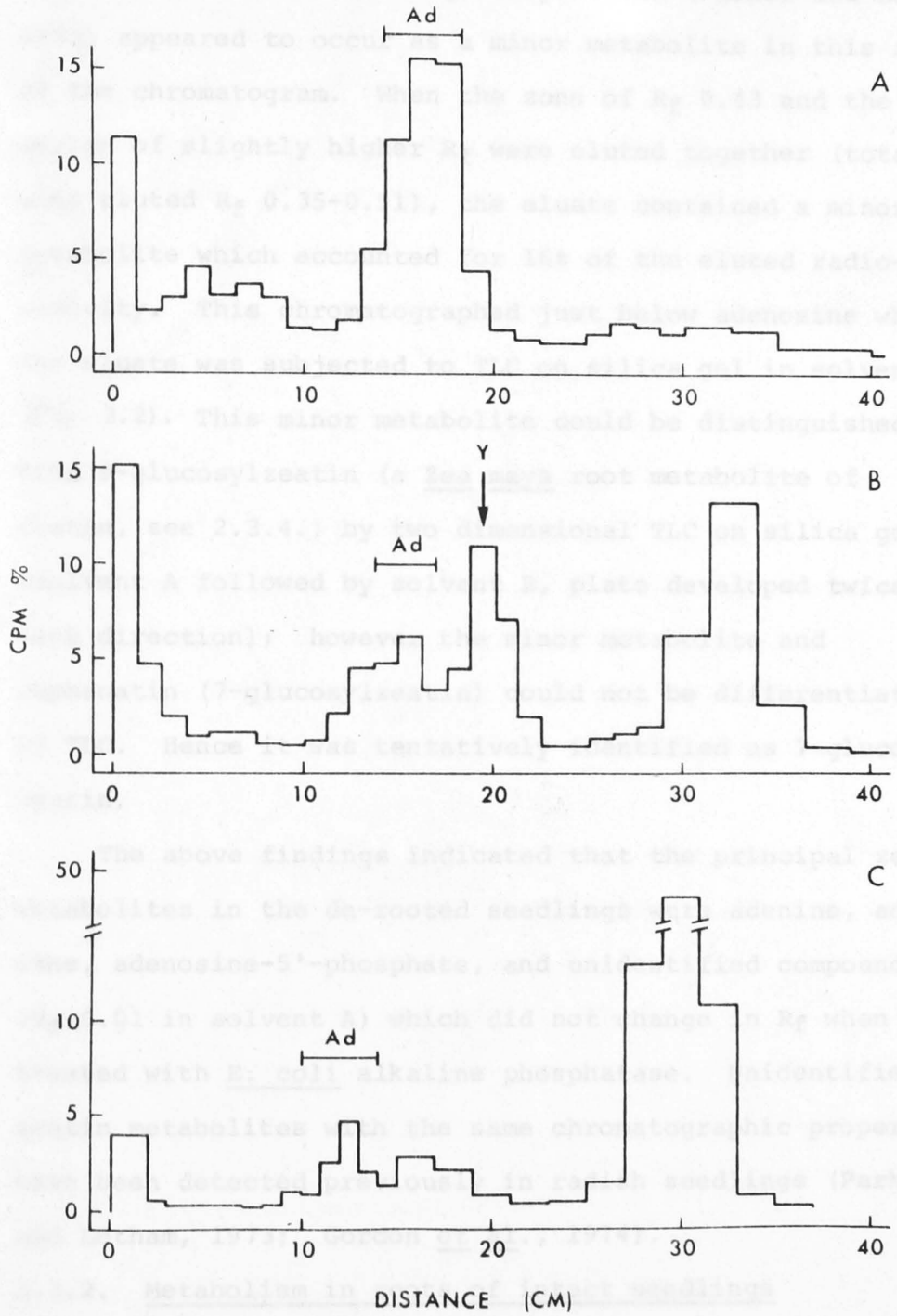
2.3.1. Metabolism in de-rooted seedlings

[G-^{3}H]Zeatin was supplied for 8 hours to the base of de-rooted seedlings which were then transferred to water and left overnight before extraction. A paper chromatogram (solvent A) of the seedling extract exhibited two prominent peaks of radioactivity which were centred at R_f values of 0.01 and 0.43 (Fig. 2.1A). An alkaline phosphatase hydrolysate prepared from the eluate of the zone at R_f 0.01 (the known location of nucleotides) was chromatographed on paper using solvent A. Two peaks of radioactivity were present on the resulting chromatogram; one was located near the origin and contained 58% of the eluted radioactivity while the other coincided with cochromatographed adenosine and represented 36% of the eluted radioactivity. TLC on silica gel (solvent A) with cochromatographed adenosine confirmed that the radioactivity of the latter zone was due to adenosine. Labelled zeatin riboside was not detected in the enzyme hydrolysate. When the eluate of the labelled zone near the origin in Fig. 2.1A was treated sequentially with periodate and cyclohexylamine (a procedure which degrades ribonucleoside-5'-phosphates to bases), 32% of the radioactivity cochromatographed with adenine (silica gel TLC, solvent A).

TLC on DEAE-cellulose and silica gel (solvents C and A respectively) established that the zone of radioactivity at R_f 0.43 (Fig. 2.1A) was largely due to adenine and adenosine; the two compounds made nearly equal

Fig. 2.1A-C. Histograms defining the distribution of radioactivity over paper chromatograms (solvent A) of extracts of de-rooted Zea mays seedlings (A), Z. mays roots (B) and cultured embryonic tissue of Z. mays (C). [G-^{3}H]Zeatin was supplied to each tissue at a concentration of 8 μM. The location of cochromatographed adenosine (Ad) is indicated on each figure; the location of metabolite Y is shown in B. Radioactivity is expressed as a percentage of the total recovered from the chromatogram.

CPM %


Ad
A
15
10
5
0
0
10
20
30
40
Y
B
Ad
CPM %
C
50
Ad
DISTANCE (CM)

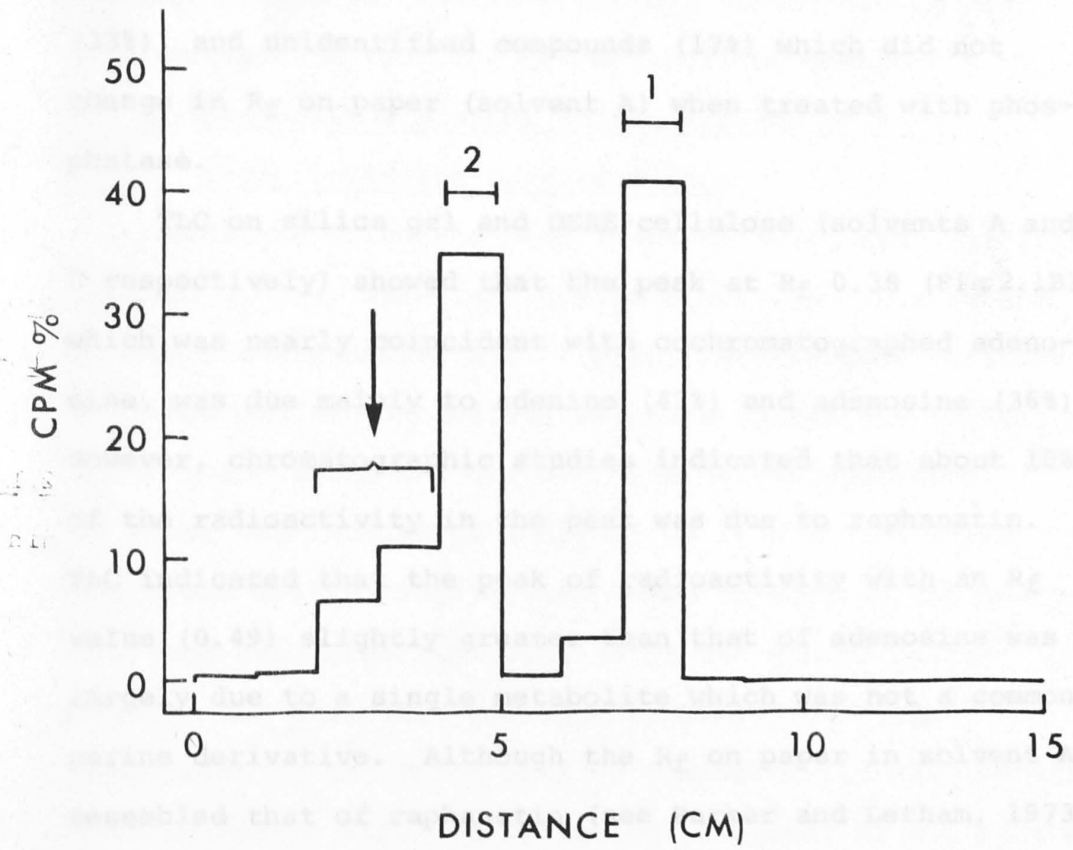
contributions. However 7-glucosylzeatin (Parker and Letham, 1973) appeared to occur as a minor metabolite in this region of the chromatogram. When the zone of R_f 0.43 and the region of slightly higher R_f were eluted together (total area eluted R_f 0.35-0.51), the eluate contained a minor metabolite which accounted for 16% of the eluted radioactivity. This chromatographed just below adenosine when the eluate was subjected to TLC on silica gel in solvent A (Fig. 2.2). This minor metabolite could be distinguished from 9-glucosylzeatin (a Zea mays root metabolite of zeatin, see 2.3.4.) by two dimensional TLC on silica gel (solvent A followed by solvent B, plate developed twice in each direction); however the minor metabolite and raphanatin (7-glucosylzeatin) could not be differentiated by TLC. Hence it was tentatively identified as 7-glucosylzeatin.

The above findings indicated that the principal zeatin metabolites in the de-rooted seedlings were adenine, adenosine, adenosine-5'-phosphate, and unidentified compounds (R_f 0.01 in solvent A) which did not change in R_f when treated with E. coli alkaline phosphatase. Unidentified zeatin metabolites with the same chromatographic properties have been detected previously in radish seedlings (Parker and Letham, 1973; Gordon et al., 1974).

2.3.2. Metabolism in roots of intact seedlings

The metabolism of zeatin by the roots of intact Zea mays seedlings was also investigated. The distribution of radioactivity over a paper chromatogram (solvent A) of

Fig. 2.2. Histogram defining the distribution of radioactivity over a silica-gel thin-layer chromatogram (solvent A) of eluate of the region R_f 0.35-0.51 on the paper chromatogram of Fig. 2.1A. Cochromatographed marker compounds were adenine (1) and adenosine (2). The radioactivity probably due to 7-glucosylzeatin is denoted by the arrow and bracket.


50
40
30
20
10
0
CPM %
1
2
0
5
10
15
DISTANCE (CM)

root extract exhibited 4 peaks (Fig. 2.1B). By phosphatase hydrolysis, periodate oxidation followed by cyclohexylamine treatment, and by paper and thin layer chromatography of the degradation products, the peak of radioactivity near the origin (R_f 0.01) was shown to be due principally to adenosine-5'-phosphate (35%), zeatin riboside-5'-phosphate (33%), and unidentified compounds (17%) which did not change in R_f on paper (solvent A) when treated with phosphatase.

TLC on silica gel and DEAE-cellulose (solvents A and C respectively) showed that the peak at R_f 0.38 (Fig. 2.1B), which was nearly coincident with cochromatographed adenosine, was due mainly to adenine (43%) and adenosine (36%). However, chromatographic studies indicated that about 10% of the radioactivity in the peak was due to raphanatin. TLC indicated that the peak of radioactivity with an R_f value (0.49) slightly greater than that of adenosine was largely due to a single metabolite which was not a common purine derivative. Although the R_f on paper in solvent A resembled that of raphanatin (see Parker and Letham, 1973), two dimensional TLC on silica gel (solvent A followed by solvent B) established that the metabolite and raphanatin were not identical; the unknown compound possessed an R_f slightly greater than that of raphanatin in this system. The new metabolite was termed compound Y and its purification and characterization are discussed below.

TLC of the eluate of the labelled zone at R_f 0.81 (Fig. 2.1B) showed that the radioactivity was due principally

to zeatin while zeatin riboside accounted for about 10% of the radioactivity.

Hence, the roots of Zea mays plants metabolized zeatin to adenosine-5'-phosphate, zeatin riboside-5'-phosphate, unidentified compounds of R_f 0.01 on paper in solvent A, adenine, adenosine, zeatin riboside, raphanatin (a very minor metabolite) and the new metabolite, compound Y.

2.3.3. Metabolites present in the sap and roots of decapitated Zea mays seedlings

Seedlings which had been 'watered' with a[G-^{3}H]zeatin solution (16 μM) were left for 36 hours, decapitated and the sap exudate collected. A paper chromatogram (solvent A) of the exudate exhibited two peaks of radioactivity, one of which cochromatographed with marker adenosine. The other was located at a higher R_f in a region where zeatin and zeatin riboside are known to occur. The adenosine peak accounted for about 7% of the eluted radioactivity and the other for approximately 70%. The composition of the large peak of radioactivity was further investigated by TLC. On silica gel (solvent A) all of the radioactivity was found to cochromatograph with authentic marker zeatin. This result was confirmed in a two-dimensional silica gel TLC system (solvent A followed by solvent B). Thus, zeatin supplied to the roots of Zea seedlings appears to be translocated largely unaltered in the sap.

The metabolites present in the root extract prepared from these seedlings (see 2.2.2.) were examined. Assess-

ment of the distribution of radioactivity over a paper chromatogram (solvent A) of the extract showed a single peak at the origin and a broad zone of radioactivity (R_f 0.30-0.45) in the region of cochromatographed adenosine. Separate eluates were prepared from the origin zone, the adenosine zone, and the region in front of the adenosine zone up to R_f 0.45. Treatment of the origin zone eluate with alkaline phosphatase caused only 5% of the radioactivity to change in R_f when rechromatographed in solvent A. This radioactivity cochromatographed with marker adenosine. These results are in sharp contrast to those obtained for the same zone from the root extract of intact seedlings (see 2.3.2.). In that zone, only 17% of the radioactivity was not changed in R_f by phosphatase hydrolysis. TLC studies of the other two eluates gave results in agreement with those obtained from the intact seedlings. Thus, the adenosine zone was found to contain approximately equal amounts of radioactivity which cochromatographed with marker adenine and adenosine (solvent B) and the higher R_f zone contained essentially only one labelled component which was chromatographically similar to the metabolite Y.

2.3.4. Metabolism in a culture of embryonic tissue

Labelled zeatin was also supplied to embryonic Zea mays tissue cultured in vitro. The distribution of radioactivity over a paper chromatogram of the tissue extract (Fig. 2.1C) resembled the profile for the root-extract chromatogram. The radioactive zone with an R_f slightly greater than that of adenosine was eluted. TLC of the

eluate indicated the radioactivity was largely due to compound Y. The identity of the metabolites in the other zones was not investigated.

2.3.5. Properties and identity of metabolite Y

Metabolite Y was purified by the procedure described in Methods. The compound was characterized by mass spectrum (Fig. 2.3) and by UV spectra (Table 2.1). The mass spectrum closely resembled that of raphanatin (Parker and Letham, 1973) but the two spectra did show significant differences in peak intensity, notably in the intensity of the peak at m/e 248. The mass spectrum of Y exhibited a molecular ion peak at m/e 381.1644 (calculated for $C_{16}H_{23}N_5O_6$:381.1648) and indicated that Y was a zeatin-hexose sugar conjugate. Below m/e 220, the mass spectrum resembled that of zeatin; the principle peaks in this m/e range in the spectrum of zeatin have been interpreted previously in terms of ion reactions and structure (Shannon and Letham , 1966). Most of the peaks above m/e 220 (Fig. 2.3) can also be assigned to particular fragment ions. If M and B represent the molecular ion of Y and the purine moiety respectively, the following structural assignments can be made:

m/e 364 : M^+-OH

m/e 361 : M^+-(H_2O + 2H) (structure I)

m/e 350 : M^+-CH_2OH (lost from the zeatin side chain)

m/e 322 : structure II

m/e 298 : protonated 9-glucosyladenine fragment ion

m/e 248 : B + C^+HOH (an ion also prominent in the

Fig. 2.3. Mass spectrum of metabolite Y taken with an AEI MS902 mass spectrometer.

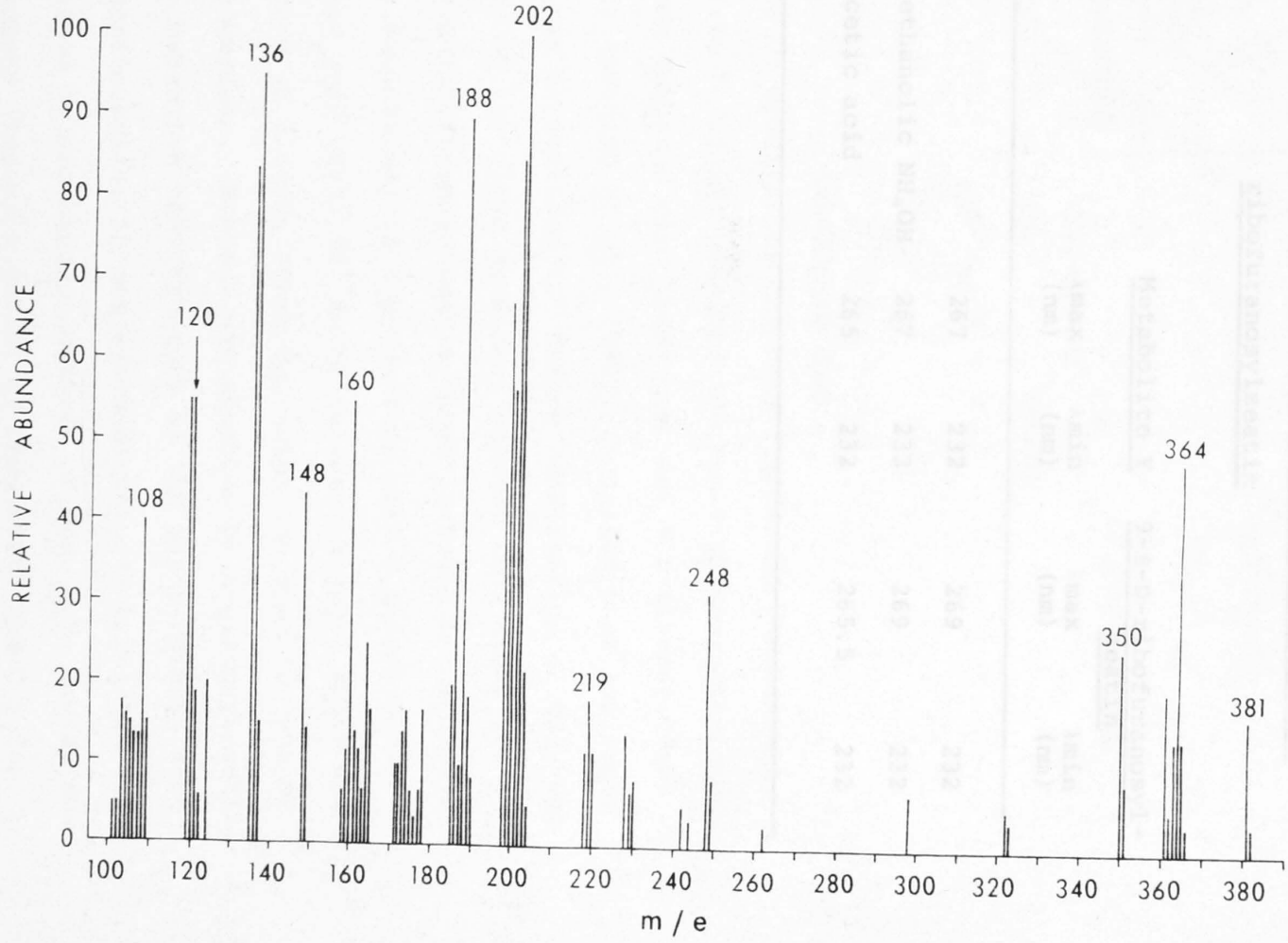

RELATIVE ABUNDANCE
100
90
80
70
60
50
40
30
20
10
0
108
120
136
148
160
188
202
219
248
350
364
381
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
m / e

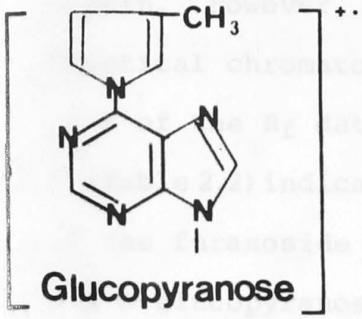
Table 2.1

Spectral characteristics of metabolite Y and 9-β-D ribofuranosylzeatin

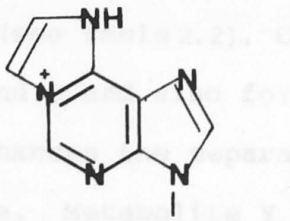
Solvent	Metabolite Y		9-β-D-ribofuranosyl-zeatin	
	λmax (nm)	λmin (nm)	λmax (nm)	λmin (nm)
ethanol	267	232	269	232
0.25 N ethanolic NH_4OH	267	233	269	232
0.1 N acetic acid	265	232	265.5	232

spectra of nucleosides, often termed "B+30" ion)

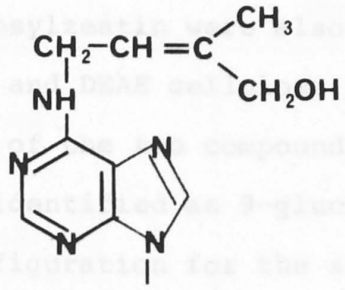
I



II



III



The UV spectra for compound Y differed considerably from those for raphanatin and the spectral characteristics of Y when compared with those recorded for disubstituted adenines (Leonard et al., 1965), indicated that the sugar moiety was at position 9. Strong confirmation of this was provided by the close agreement between the values for Y and 9-β-D-ribofuranosylzeatin shown in Table 2.1. Hydrolysis of Y in aqueous solution by heating with a polystyrene sulphonic acid resin (H^+ form) released a sugar which was identified as glucose after TLC using the specific enzyme glucose oxidase. Hence Y was shown to be 9-glucosylzeatin.

To establish the structure of the sugar moiety, 9-β-D-glucopyranosylzeatin and 9-β-D-glucofuranosylzeatin were synthesised by unambiguous methods by Drs. D.E. Cowley and J.K. MacLeod (Research School of Chemistry, A.N.U.) for comparison with the metabolite Y. TLC studies clearly

demonstrated that Y differed from 9-β-D-glucofuranosyl-zeatin. However, Y and 9-β-D-glucopyranosylzeatin exhibited identical chromatographic behaviour (see Table 2.2). Comparison of the R_f data for solvents L and J and also for K and I (Table 2.2) indicates that borate enhances the separation of the furanoside from the pyranoside. Metabolite Y and 9-β-D-glucopyranosylzeatin were also indistinguishable by TLC on polyamide and DEAE cellulose. In addition, the UV and mass spectra of the two compounds were identical and therefore Y was identified as 9-glucopyranosylzeatin with a probable β configuration for the anomeric linkage (structure III).

2.3.6. Comparison of the metabolites formed from zeatin and 6-benzylaminopurine in derooted seedlings of Zea mays

Two identical sets of 12-day-old, derooted seedlings were selected; one set was supplied with a 10 μM solution of [G-^{3}H]zeatin and the other with a 10 μM solution of the synthetic cytokinin [G-^{3}H]6-benzylaminopurine (BAP; specific activity 19.0 mCi/mmole). The two sets of seedlings were allowed to take up the hormone containing solutions for 70 hours and then extracted. The experimental conditions and extraction methods used have been described previously (see 2.2.1.). Aliquots of the two extracts were subjected to silica gel TLC (solvent A) and the distribution of radioactivity over each chromatogram was assessed. Adenosine and zeatin or BAP were cochromatographed with the extracts to serve as UV visible markers.

Table 2.2

TLC comparison of metabolite Y with two zeatin derivatives

TLC system	R_f values		
	Y	9-β-D-gluco-furanosyl-zeatin	9-β-D-gluco-pyranosyl-zeatin
Silica gel - solvent A	0.16	0.26	0.16
Borate impregnated silica gel - solvent I	0.53	0.35	0.53
Borate impregnated silica gel - solvent J	0.55	0.33	0.56
DEAE cellulose sprayed with 0.05 M borate - solvent I	0.55	0.46	0.55
Silica gel - solvent L	0.68	0.71	0.68
Silica gel - solvent K	-	0.70	0.67

The histograms of Fig. 2.4A,B indicate definite differences in the metabolism of zeatin and BAP by the corn seedlings. Approximately 30% of the eluted radioactivity from the chromatogram of the extract of seedlings supplied with BAP cochromatographed with authentic BAP whereas on the other chromatogram less than 4% of the eluted radioactivity co-chromatographed with authentic zeatin. A more detailed examination of the identities of the metabolites present in each extract was undertaken using two-dimensional TLC on silica gel (solvent A, solvent B). This procedure further emphasised the differences in metabolism of the two cytokinins by the corn seedlings. Adenine and adenosine were cochromatographed with each extract together with the parent cytokinins and their derivatives as indicated in Table 2.3. In this table, radioactivity cochromatographing with each marker is listed and that eluted from the remainder of the chromatogram is termed unidentified radioactivity. The data relating to the nucleotide metabolites were obtained from TLC on DEAE cellulose layers (solvent: methanol-water, 1:1 v/v), a chromatographic system in which nucleotides are known to exhibit very low mobilities. These data were confirmed in studies using the enzyme alkaline phosphatase.

Thus, although similar amounts of the cytokinins (0.16 and 0.21 μmoles of zeatin and BAP, respectively) were taken up by each set of seedlings, those supplied with zeatin metabolised a much greater proportion of the cytokinin. The major zeatin metabolites identified by

Fig. 2.4A,B. Histograms showing the distribution of radioactivity after silica gel TLC (solvent A) of crude extracts of de-rooted Zea mays seedlings which had been supplied with ^{3}H labelled BAP (A) and zeatin (B) under identical conditions. The barred lines indicate the location of the cochromatographed markers adenosine (Ados), 6-benzylaminopurine (BAP) and zeatin (Z).

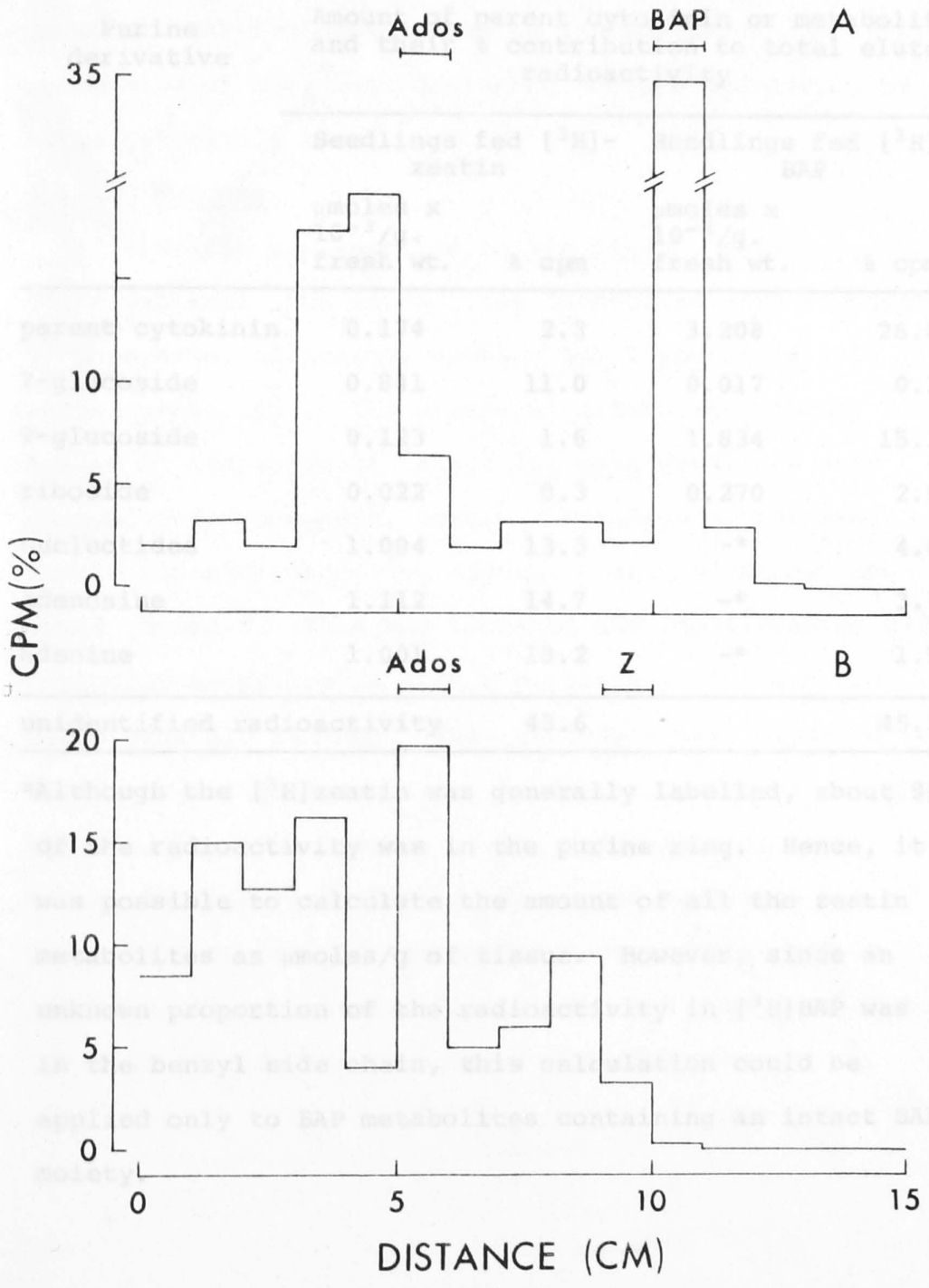

Ados
BAP
A
35
10
5
0
CPM(%)
Ados
Z
B
20
15
10
5
0
0
5
10
15
DISTANCE (CM)

Table 2.3

TLC comparison of zeatin and BAP metabolites

Purine derivative	Amount of parent cytokinin or metabolite and their % contribution to total eluted radioactivity			
	Seedlings fed [^{3}H]-zeatin		Seedlings fed [^{3}H]-BAP	
	µmoles x 10^{-3}/g. fresh wt.	% cpm	µmoles x 10^{-3}/g. fresh wt.	% cpm
parent cytokinin	0.174	2.3	3.208	26.8
7-glucoside	0.841	11.0	0.017	0.3
9-glucoside	0.123	1.6	1.834	15.3
riboside	0.022	0.3	0.270	2.5
nucleotides	1.004	13.3	-*	4.0
adenosine	1.112	14.7	-*	3.7
adenine	1.001	13.2	-*	1.9
unidentified radioactivity		43.6		45.5

*Although the [^{3}H]zeatin was generally labelled, about 95% of the radioactivity was in the purine ring. Hence, it was possible to calculate the amount of all the zeatin metabolites as µmoles/g of tissue. However, since an unknown proportion of the radioactivity in [^{3}H]BAP was in the benzyl side chain, this calculation could be applied only to BAP metabolites containing an intact BAP moiety.

chromatography were zeatin 7-glucoside, nucleotides, adenine and adenosine which were formed in very similar amounts. BAP-9-glucoside was by far the major identified metabolite of BAP, degradation to adenine and adenosine being relatively minor.

2.4.1. Summary

[^{3}H]Zeatin was supplied to Zea mays L. seedlings with roots excised; the metabolites identified were adenosine-5'-phosphate, adenosine, adenine and 7-glucosylzeatin (a minor metabolite). The principal metabolites formed from zeatin by the roots of intact Zea mays seedlings were adenosine-5'-phosphate, zeatin riboside-5'-phosphate, zeatin riboside, adenine, adenosine and an unknown compound termed Y. This was isolated and identified as 9-β-D-glucopyranosylzeatin with a probable β-configuration for the glycosidic linkage. This glucoside also appeared to form from zeatin in cultured embryonic tissue of Zea mays. Also, comparative studies of the metabolism of zeatin and 6-benzylaminopurine (BAP) in de-rooted seedlings are reported. The results presented indicate that BAP is considerably more stable than zeatin and much less susceptible to degradation to adenine and adenosine.

3.1. INTRODUCTION

3.1.1. The leaves of blue lupin seedlings show sequential abscission and senescence. The lupin leaf consists of three regions, namely, the petiole, the leaflets and the intermediate region which has been termed the pulvinar region. On the intact plant, each leaflet abscises independently at an abscission zone within its pulvinus. Much later the petiole also abscises (Carr and Burrows, 1967).

Kinetin applied to the pulvinar region of detached lupin leaves delays leaflet abscission (Burrows and Carr, 1967). Uptake of kinetin in the transpiration stream of detached leaves delays abscission both in these leaves and in pulvinar explants derived from them. This suggests that kinetin supplied through the transpiration stream reaches the pulvinar region and as a consequence retards abscission (Burrows and Carr, 1967). If endogenous cytokinins exert a similar regulatory role in the intact leaf, abscission may be associated with a decline in the capacity of the leaf to derive cytokinins from the xylem sap. Alternatively, the leaf may develop an enhanced ability to inactivate cytokinins or to antagonize their activity as abscission-delaying agents.

Clearly the above work could be extended by investigations of the translocation and metabolism of labelled, natural cytokinins, such as zeatin, in lupin seedlings. Such studies would also be relevant in relation to the phenomenon of sequential leaf senescence which is observed in lupin. However, before translocation studies would be

worthwhile, considerable knowledge of the metabolism of zeatin in lupin seedlings would be required. In this chapter, a study is reported of the metabolites formed from zeatin in seedlings of Lupinus angustifolius.

3.2. EXPERIMENTAL

3.2.1. Uptake of [^{3}H]zeatin by lupin seedlings and tissue extraction

[G-^{3}H]Zeatin (see 2.2.1.) at a concentration of 10 µM was mixed with unlabelled zeatin (Calbiochem) to give a 100 µM aqueous solution of the phytohormone. The stems of 9-day-old de-rooted lupin seedlings (Lupinus angustifolius cv. New Zealand blue) were placed in this solution and left for 21 hours in continuous fluorescent light and a gentle air current.

Sections of the seedlings were then extracted with 80% methanol as described previously (see 2.2.1.). The evaporated extracts were dissolved in 50% ethanol (0.6 gm fresh wt. tissue / 1.0 ml) for chromatography.

3.2.2. Preparation of sap extract from lupin seedlings

Lupin seedlings were grown for 17 days in sand and then deprived of water for 48 hours. They were then 'watered' with 100 mls of 2 mM phosphate buffer (pH 6.0) which contained [G-^{3}H]zeatin at a concentration of 12 µM. The seedlings were decapitated mid-way between the sand and the cotyledons after 9 hours. The exudate which appeared soon after decapitation was collected on filter paper and discarded because it probably contained material derived from ruptured cells. The droplets of sap which subsequently formed were collected over 4 days on small filter paper wedges. The filter paper was placed in 50% methanol after each collection. The combined eluates were evaporated and taken up in 200 µl of 50% methanol for

chromatography. This procedure was repeated with a second batch of seedlings using a solution which was 35 μM with respect to labelled zeatin.

The second batch of seedlings were left for 24 hours after completion of the sap collection and then removed from the sand and washed several times in distilled water. The stems were excised from each seedling and the root tissue was extracted in 80% methanol as previously described (2.2.1.). The extract was evaporated to dryness and taken up in 50% ethanol for chromatography.

3.2.3. Chromatographic and electrophoretic methods

(i) Chromatography. Chromatographic materials which have not been described previously (see 2.2.3.) are detailed below. Additional TLC layers used were CAMAG silica gel and SERVA cellulose (both 0.25 mm thick). Apart from the addition of Woelm green fluorescent indicator (0.4% by wt.), the CAMAG layers were prepared in the usual way. The CAMAG layer was used in preference to MERCK PF_{254} because it gave an improved separation of zeatin from dihydrozeatin and of zeatin riboside from dihydrozeatin riboside when developed in solvent H. In initial attempts to separate these cytokinins, numerous solvents were tested, but ethyl acetate-ethanol (9:1, v/v) was the only solvent system which suggested the possibility of a separation. Many similar solvents were then tested and the best separation was obtained with methyl acetate-ethanol (9:1 v/v). The separation was further improved and streaking suppressed by the addition of the water scavenger, 2,2 dimethoxypro-

pane, and a few drops of formic acid. Data on the mobilities of zeatin and related compounds in this solvent and also in solvent G is provided in Table 3.1. The cellulose layers were prepared by blending SERVA cellulose (19 g) and Woelm green fluorescent indicator (150 mg) with water (100 ml) at high speed in a Waring blender. This procedure was necessary in order to disperse the indicator evenly throughout the layer.

Solvent systems used in addition to those already detailed (2.2.3.) were (proportions v/v):

D. water-saturated n-butanol, atmosphere of ammonia

E. methyl ethyl ketone-acetic acid-water (8:2:4)

F. water-saturated n-butanol, saturated with $Na_2B_4O_7$

G. chloroform-methanol (9:1)

H. methyl acetate-ethanol-2,2-dimethoxypropane (90:10:1) with 5 drops of formic acid per 100 ml. The formic acid and dimethoxypropane were added to the tank immediately before use.

(ii) Electrophoresis. All high voltage electrophoresis (HVE) was carried out on an apparatus manufactured by Paton Industries, Adelaide, and Whatman 3 MM paper was used. Marker compounds, appropriate for the pH conditions, were spotted onto each electrophoretogram to assess endosmosis and relative mobility. The buffer systems used were:

A. 0.025 M phosphate at pH 10.2

B. 0.025 M phosphate at pH 9.2

C. 0.025 M phosphate at pH 7.05

D. 0.025 M phosphate at pH 6.0

E. 0.025 M triethylamine brought to pH 10.0 with

Table 3.1.

Chromatographic characteristics of zeatin and related compounds on silica gel TLC. The values listed are the distances travelled (cms) after double development. The distance between the origin and the front was 16.0 cms; CAMAG silica gel was used.

Compound	Solvent G*	Solvent H
zeatin	5.7	5.2
cis-zeatin	6.9	5.5
dihydrozeatin	5.5	4.2
zeatin riboside	4.2	6.8
cis-zeatin riboside	5.0	7.0
dihydrozeatin riboside	4.3	6.0

*These distances were derived from a two-dimensional chromatogram developed with solvent H in the first dimension. The layer had been pre-washed in solvent A and dried before use.

acetic acid

F. 0.025 M borate at pH 9.2

G. 0.05 M bicarbonate at pH 10.0

H. 0.05 M formate at pH 3.15

All electrophoretograms were run at a potential of 40 volts/cm.

3.2.4. Characterization of chromatographic fractions and purified metabolites

(i) Degradative methods. Phosphatase hydrolysis and periodate oxidation procedures, as described in 2.2.4., were used in studies of eluates from potential nucleotide regions of paper chromatograms.

Enzymic studies of the nature of the anomeric linkage of the purified zone 3 metabolite, L 3/C, involved the use of the enzymes α- and β-glucosidase. Sigma α-glucosidase type I from yeast (1.0 mg) was dissolved in 0.05 M phosphate buffer (pH 6.7, 3.0 ml) and aliquots (50 µl) used in each incubation (3 hours at 35°C). Sigma β-glucosidase (1.0 mg) was dissolved in 0.03 M acetate buffer (pH5.3, 3.0 ml) and 50 µl aliquots used in each incubation (3 hours at 35°C). Aliquots from incubations were chromatographed on PF_{254} silica gel layers with authentic zeatin as marker (solvent A) to determine the effects of the enzyme.

The specific enzyme, L-amino acid oxidase (Boehringer), was used to determine the configuration of the amino acid moiety conjugated to the purine ring of metabolite L 2. The metabolite (1 µg) was incubated with the enzyme solution (2 µl containing 2 µg) in a Tris-HCl buffer (30 µl, 0.05 M,

pH 7.3) for 6 hours at 30°C. Prior to this, the enzyme had been incubated with [^{14}C]D-alanine to check for the presence of D-amino acid oxidase activity. TLC on silica gel (solvent B) was used to assess the effect of the enzyme on the metabolite and [^{14}C]D-alanine.

(ii) Determination of mass and UV spectra. UV and mass spectra of underivatized metabolites were determined as described previously in 2.2.7. The TMS-derivative of metabolite L2 was prepared by dissolving a sample (10 µg) of the compound with pyridine (10 µl) and adding 100 µl of a mixture of bis-trimethylsilyltrifluoroacetamide and trimethylchlorosilane (99:1). The solution was heated in a stoppered vial at 100°C for 2 hours. The product was not volatile enough for GLC-mass spectrometry but a mass spectrum was obtained by evaporating an aliquot (30 µl) of the solution onto the direct inlet probe of the AEI MS902 mass spectrometer, removing the reagents under a stream of dry nitrogen, and then running the spectrum in the normal way.

3.2.5. Purification of the major metabolite in paper chromatogram zone 2

Extract equivalent to 17 g of segment A tissue (see Results 3.3.) was subjected to preparative paper chromatography (solvent A, 15 sheets). The zone containing the metabolite (R_f 0.17-0.21) was located accurately on one chromatogram from the distribution of radioactivity along a narrow strip. This zone and the corresponding zones from the other chromatograms were each eluted exhaustively

by allowing 0.15 M acetic acid to flow down them. The combined eluates were evaporated in vacuo (<40°C) and the residue was dissolved in 50% ethanol (6 ml) for preparative TLC on silica gel layers. After development in solvent A, the metabolite was again located by counting a narrow strip from one of the plates. The peak of radioactivity was coincident with a medium intensity UV absorbing zone (R_f 0.12-0.16). A small column was packed with the silica gel from these zones and eluted with 80% methanol. The eluate obtained was evaporated (<40°C) and taken up in 80% methanol for further preparative TLC on silica gel (solvent B). This procedure yielded a single UV absorbing zone (R_f 0.20) with more than 80% of the radioactivity. The residue obtained by evaporation of the 80% methanol eluate of this zone was dissolved in water and the solution (1.0 ml, pH 3) was percolated through a column of cellulose phosphate (NH_4^+ form equilibrated to pH 3.0, 4.0 ml) which was then washed with water at pH 3.0 (24 ml) and finally eluted with 0.3 M NH_4OH (36 ml). More than 75% of the radioactivity applied to the column was detected in the NH_4OH eluate which was evaporated and the residue dissolved in 600 µl of redistilled ethanol. The final purification step involved paper chromatography on washed paper in solvent D. Owing to inadequate separation after the first run, the chromatogram was developed a second time. The resulting major UV absorbing zone (R_f 0.40) and an identical blank zone were cut out as strips and eluted with 60% redistilled ethanol. Purified zone 2 metabolite (about 200 µg) thus obtained

was used for chemical characterization by UV and mass spectra. The metabolite was subsequently termed L 2.

3.2.6. Purification of metabolites in paper chromatogram zone 3

Strips containing the peak of radioactivity termed zone 3 (Fig. 3.2A) were cut out from the paper chromatograms on which the initial preparative separation of zone 2 had also been achieved. The combined eluates (0.15 M acetic acid) from these strips were evaporated *in vacuo* (<40°C) and dissolved in 50% ethanol (6.0 ml). This solution was loaded onto seven 0.5 mm thick silica gel TLC plates which were developed in solvent A. Zones from a narrow strip off one plate were eluted for liquid scintillation counting. This procedure showed that >76% of the radioactivity was contained in a 2.0 cm wide strip centred on a UV-absorbing zone at R_f 0.19. A small glass column was packed with the silica from these zones and eluted with 80% methanol. The evaporated eluate was dissolved in 4.0 ml water, adjusted to pH 3.0 and chromatographed on a column of cellulose phosphate (NH_4^+ form equilibrated to pH 3.0; 6 ml) which was washed with water at pH 3.0 (36 ml) and eluted with 0.3 M NH_4OH (54 ml). Most of the radioactivity was eluted by the NH_4OH which was evaporated and the residue was taken up in 50% methanol (0.5 ml). Further purification was achieved using a two-dimensional paper chromatography system (washed Schleicher and Schull 2040B, solvent E followed by solvent D) which resolved the mixture into six UV absorbing spots (Fig. 3.1.). These were designated L 3/1, L 3/2, L 3/3,

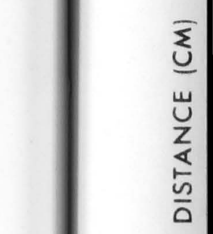


Fig. 3.1. The location on a two-dimensional paper chromatogram of the UV absorbing components contained in a fraction derived from zone 3 of a paper chromatogram of segment A extract. L 3/1 appeared to be a mixture of two incompletely resolved metabolites (see text).

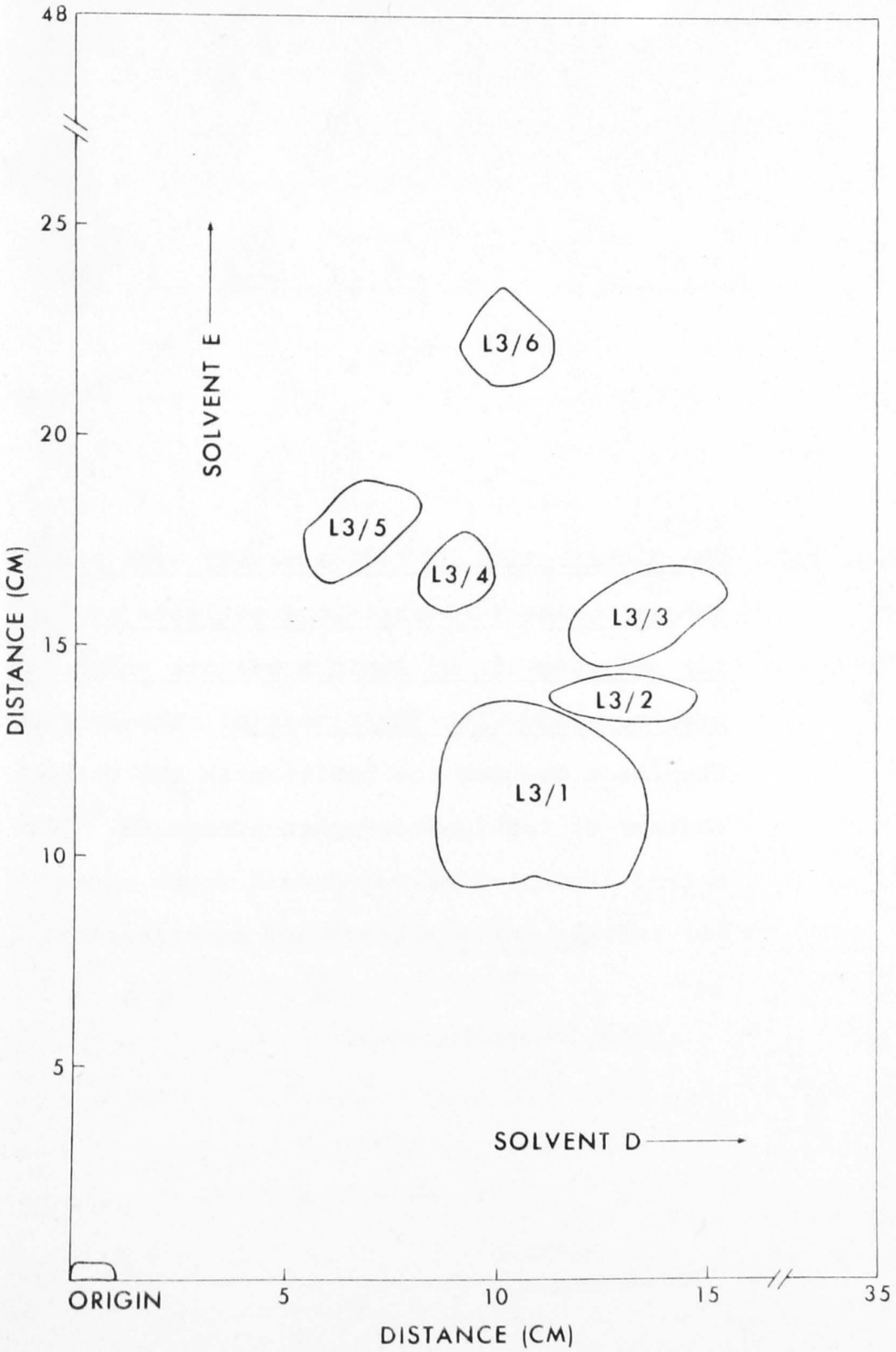
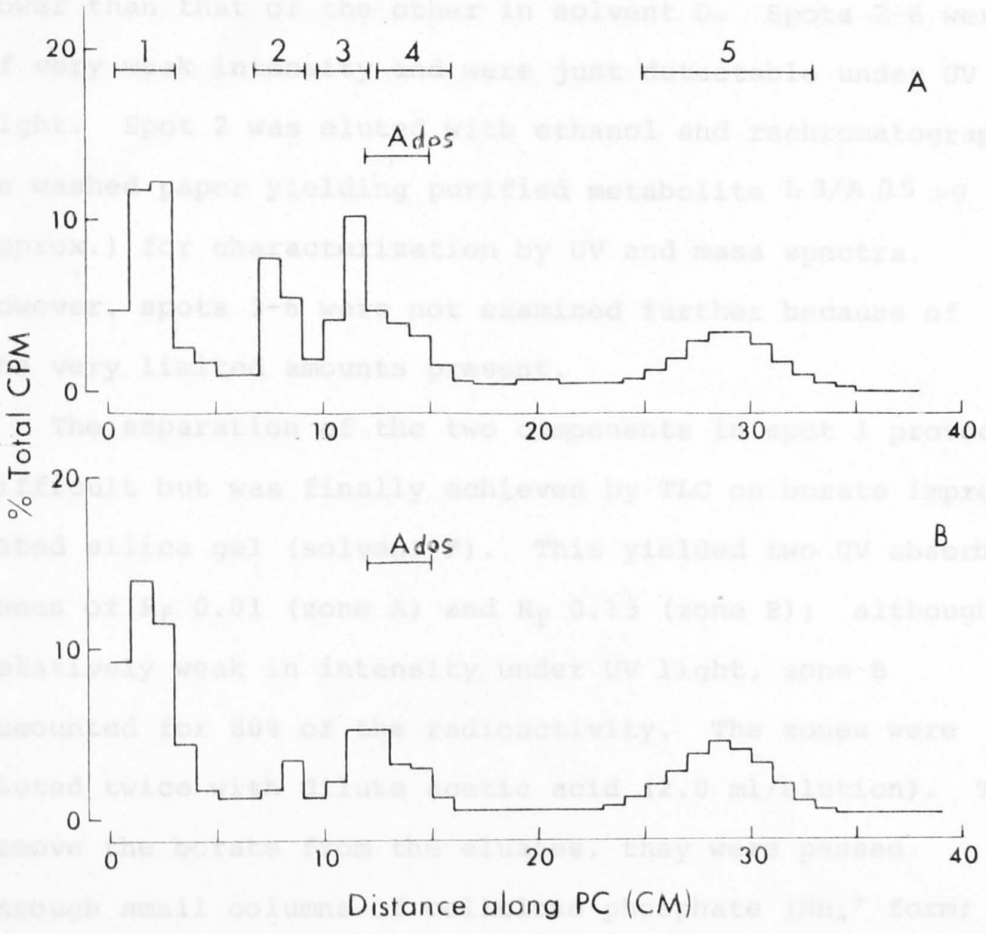

48
25
20
15
10
5
DISTANCE (CM)
SOLVENT E
L3/6
L3/5
L3/4
L3/3
L3/2
L3/1
SOLVENT D
ORIGIN
5
10
15
35
DISTANCE (CM)

Fig. 3.2. The distribution of radioactivity over paper chromatograms (solvent A) of extracts of leaf (A) and stem (B) of lupin seedlings which had been supplied with [G-^{3}H]zeatin. The barred line Ados denotes the position on the chromatograms of cochromatographed adenosine. The barred lines 1-5 of 2 A denote zones eluted for further investigation and purification.


20
1
2
3
4
5
A
Ados
10
0
% Total CPM
0
10
20
30
40
20
Ados
B
10
0
0
10
20
30
40
Distance along PC (CM)

L 3/4, L 3/5 and L 3/6 and their relative radioactivities were 26.40, 7.50, 2.30, 0.13, 0.03 and 1.00 respectively. Spot L 3/1 appeared to be a mixture of two incompletely separated metabolites which differed greatly in their intensity. The R_f of the intense component was slightly lower than that of the other in solvent D. Spots 2-6 were of very weak intensity and were just detectable under UV light. Spot 2 was eluted with ethanol and rechromatographed on washed paper yielding purified metabolite L 3/A (15 µg approx.) for characterization by UV and mass spectra. However, spots 3-6 were not examined further because of the very limited amounts present.

The separation of the two components in spot 1 proved difficult but was finally achieved by TLC on borate impregnated silica gel (solvent F). This yielded two UV absorbing zones of R_f 0.01 (zone A) and R_f 0.13 (zone B); although relatively weak in intensity under UV light, zone B accounted for 80% of the radioactivity. The zones were eluted twice with dilute acetic acid (2.0 ml/elution). To remove the borate from the eluates, they were passed through small columns of cellulose phosphate (NH_4^+ form; equilibrated to pH 3.0) which were washed with water (pH 3.0) and eluted with NH_4OH. The NH_4OH eluates thus obtained were taken to dryness <u>in vacuo</u> (<40°C) and each residue dissolved in 50% ethanol (200 µl) for final purification by paper chromatography on washed paper (solvent D).

By the above procedure, the zone of R_f 0.01 yielded a single UV-absorbing spot which was eluted with 50% ethanol

to give purified metabolite L 3/B (80 µg approx.). However, the zone of R_f 0.13 yielded two UV absorbing spots, R_f 0.28 and 0.34. The former (R_f 0.28) which was the more intense was eluted with ethanol to yield purified metabolite L 3/C (40 µg approx.), while the latter gave purified metabolite L 3/D (15 µg approx.). These metabolites were characterized by UV and mass spectra.

3.3. RESULTS

3.3.1. Chromatographic characterization of metabolites in shoot extracts

3.3.1.1. Paper chromatography of crude extracts.

Nine-day-old de-rooted lupin seedlings were supplied with tritium-labelled zeatin as described in section 3.2.1. Lupin seedlings of this age have only two fully developed leaves attached to the main stem at the tip of which is a cluster of young emerging leaves. Below the developed and emerging leaves is a pair of large fleshy cotyledons. Before extraction, the seedlings were divided into three segments, (a) the shoot above the cotyledons, (b) the shoot below the cotyledons, and (c) the cotyledons themselves. Segment (a) consisted mainly of leaf tissue but approximately 20% of the fresh weight was stem. Henceforth, extracts of segments (a), (b) and (c) may be referred to as leaf, stem and cotyledon extracts, respectively, for convenience.

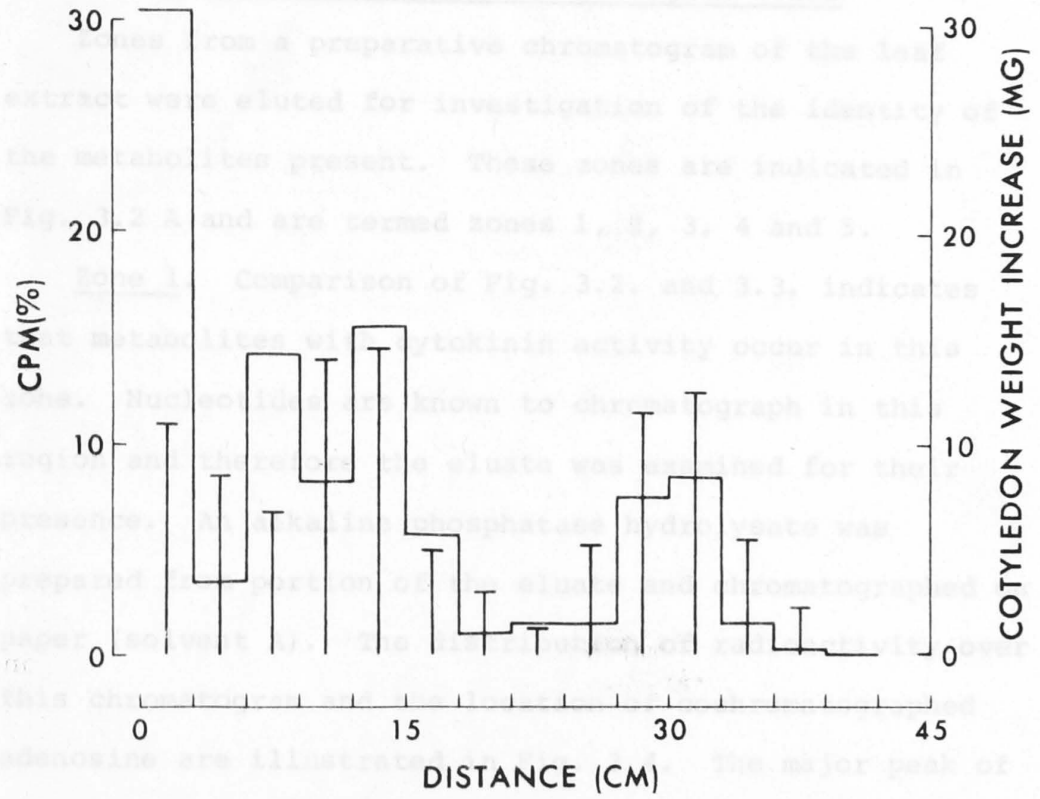
The distribution of radioactivity over paper chromatograms (solvent A) of extracts of segments (a) and (b) is presented in Fig. 3.2 A and B. The chromatograms of these extracts showed four peaks of radioactivity at the R_f values of 0.05, 0.20, 0.30 and 0.70, as well as a pronounced shoulder of radioactivity associated with the peak at R_f 0.30. The major difference between the two distributions is that the peak at R_f 0.20 for leaf extract is relatively more intense than it is for stem extract. Extracts were also prepared from the leaves of seedlings induced to wilt

during the final stages of the uptake of zeatin. These extracts and also those prepared from the cotyledons of unwilted seedlings yielded chromatograms with a radioactivity distribution similar to that of Fig. 3.2 A. However, in the case of cotyledons, the level of radioactivity present was approximately one tenth of that present in the extracts of the other two segments.

3.3.1.2. Cytokinin activity in a leaf extract.

The distribution of cytokinin activity over paper chromatograms of leaf extracts was also determined. A small set of de-rooted seedlings were supplied with labelled zeatin and a second set (control seedlings) were supplied with water under conditions identical to those described in 3.2.1. Extracts of the zeatin and control seedlings were prepared and aliquots chromatographed on the same paper (solvent A). The two chromatograms were divided into zones of identical R_f for the detection of cytokinin activity using the radish cotyledon expansion bioassay (Letham, 1971). The chromatogram of the control extract yielded cotyledon weights which served as a basis for calculating the increments in cotyledon weight induced by the zones of the other chromatogram in which the labelled metabolites were separated. A narrow strip was removed from the edge of the labelled chromatogram for determination of the distribution of radioactivity. The relationship between cytokinin activity and radioactivity is illustrated in Fig. 3.3. in which it can be seen that three major peaks of radioactivity (R_f 0.04, 0.34 and 0.75) coincide with

Fig. 3.3. The distribution of cytokinin activity relative to radioactivity over a paper chromatogram (solvent A) of a leaf extract of lupin seedlings which had been supplied with [G-^{3}H]zeatin. The histogram represents the % of total eluted radioactivity present in each 3.0 cm zone of the chromatogram and the barred vertical lines represent the mean weight increments induced per radish cotyledon. The chromatogram was loaded with the equivalent of 0.13 g fresh weight of tissue. The increment induced in the presence of 14 μM zeatin riboside was 15 mg. The radish cotyledons were placed directly on the paper chromatogram zones.


30
20
10
0
CPM(%)
30
20
10
0
COTYLEDON WEIGHT INCREASE (MG)
0
15
30
45
DISTANCE (CM)

prominent peaks of cytokinin activity. However, the prominent radioactive peak at R_f 0.20 exhibited only weak cytokinin activity. Similar results were obtained when the assay was repeated.

3.3.2. Metabolites in paper chromatogram zones

Zones from a preparative chromatogram of the leaf extract were eluted for investigation of the identity of the metabolites present. These zones are indicated in Fig. 3.2 A and are termed zones 1, 2, 3, 4 and 5.

Zone 1. Comparison of Fig. 3.2. and 3.3. indicates that metabolites with cytokinin activity occur in this zone. Nucleotides are known to chromatograph in this region and therefore the eluate was examined for their presence. An alkaline phosphatase hydrolysate was prepared from portion of the eluate and chromatographed on paper (solvent A). The distribution of radioactivity over this chromatogram and the location of cochromatographed adenosine are illustrated in Fig. 3.4. The major peak of radioactivity (>70% of the total) which resulted from the phosphatase hydrolysis occurred at R_f 0.70, the probable location of zeatin riboside in this chromatographic system. Two other small peaks were present, one cochromatographing with marker adenosine and the other at the origin. In a two-dimensional system involving paper chromatography (solvent A) followed by high voltage electrophoresis (HVE) using buffer F, the result above was confirmed when the bulk of the radioactivity was found to run with marker zeatin riboside. The procedures used so far do not distinguish between

Fig. 3.4. The distribution of radioactivity over a paper chromatogram of the zone 1 eluate (see Fig. 3.2 A) after treatment with alkaline phosphatase. The barred line Ados denotes the location on the chromatogram of cochromatographed adenosine.

Ados
CPM(%)
20
15
10
5
0
0
10
20
30
40
DISTANCE (CM)

zeatin riboside and dihydrozeatin riboside. A TLC system was developed in which the free bases zeatin and dihydrozeatin were separated (see section 3.2.3.). Hence, it was necessary to oxidise the nucleotides in the zone 1 eluate to the corresponding free bases using periodate (see 2.2.4.). Only nucleoside 5'-phosphates are degraded by this procedure. TLC of the product with adenine, dihydrozeatin and zeatin markers in the system described, indicated that the relative amounts of radioactivity which cochromatographed with these markers were approximately 1.0, 3.4 and 9.5 respectively. Thus, from chromatographic evidence, the radioactivity in the zone 1 eluate is largely zeatin riboside 5'-phosphate together with lesser amounts of dihydrozeatin riboside and adenosine 5'-phosphates and metabolites unchanged in R_f by phosphatase treatment.

Zone 2. This peak of radioactivity which appears to be associated with weak cytokinin activity (c.f. Fig. 3.2 and 3.3), occurs at a different R_f to that of any identified metabolite of zeatin. However, a minor unidentified metabolite of zeatin in radish seedlings has been reported to chromatograph in this region (Parker and Letham, 1973a). Hypoxanthine is the only common purine with approximately the same R_f in this chromatographic system. However, the possibility that the metabolite of zone 2 was hypoxanthine was eliminated by TLC on silica gel in two solvent systems. In solvent A, the metabolite and hypoxanthine chromatographed at R_f values of 0.26 and 0.33 respectively and in solvent B at R_f values of 0.30 and 0.50. The behaviour of

the metabolite on ion exchange columns of cellulose phosphate (NH_4^+ form; equilibrated to pH 3) and DEAE cellulose (HCO_3^- form; see 3.2.5.) was next determined. In both systems, over 90% of the radioactivity was retained on the ion exchange cellulose column after washing. In a two-dimensional silica gel TLC system (solvent A, solvent B), the bulk of the radioactivity was found to be coincident with a weak UV absorbing spot. Spots from several such chromatograms were tested in the radish cotyledon expansion bioassay for cytokinins and were found to be weakly active. Since this metabolite was new and it appeared to have cytokinin activity, its isolation and identification (section 3.2.5.) were undertaken. This new metabolite was termed L 2.

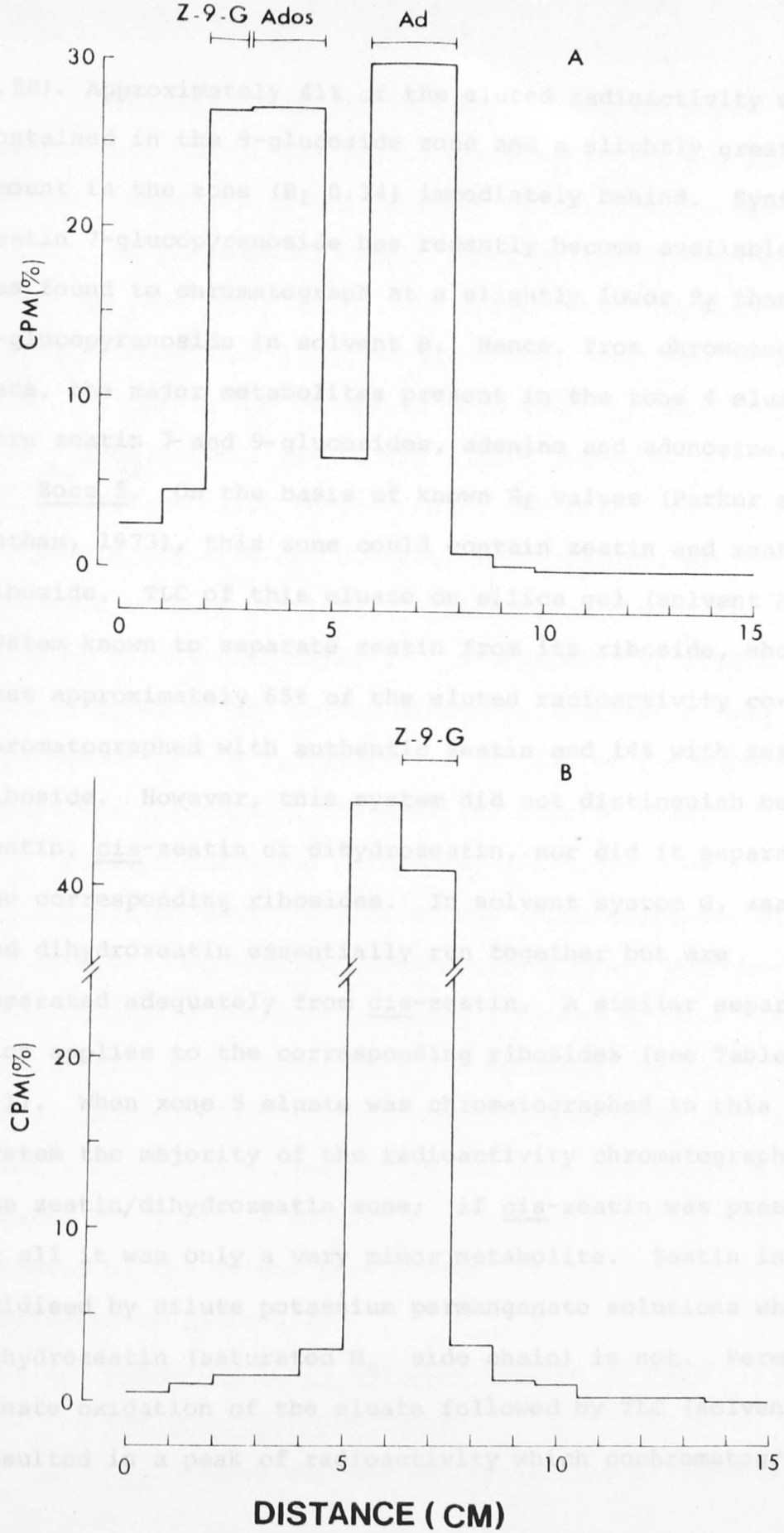
Zone 3. The metabolite complex present in this zone possesses cytokinin activity (see Fig. 3.3). The metabolites, which possess an R_f value slightly less than that of adenosine on paper in solvent A, differ chromatographically from any previously identified zeatin metabolites. Zeatin 7- and 9-glucosides have similar R_f values, but these slightly exceed that of adenosine on paper in solvent A. TLC on silica gel in solvent B resulted in a broad peak of radioactivity over the R_f range 0.3-0.4, but when a similar chromatogram was developed in solvent A, the radioactivity was resolved into three separate peaks at R_f 0.22, 0.40 and 0.59. These peaks accounted for 59, 16 and 14% respectively of the eluted radioactivity. The behaviour of this metabolite complex was also examined on ion exchange

celluloses and it was found that 95% of the radioactivity was held on cellulose phosphate (NH_4^+ form, pH 3) but none was retained by DEAE cellulose (HCO_3^- form). All of the metabolites present in this complex were unknown and hence of considerable interest, but only the metabolites in the major peak of radioactivity (R_f 0.22, silica gel and solvent A) were present in a sufficient amount to warrant an attempted purification. The purification was undertaken by the procedures detailed in experimental section 3.2.6. and in all, four metabolites, L/3/A, /B, /C and /D were purified.

Zone 4. This is the region to which the 7- and 9-glucosides of zeatin are known to move during paper chromatography in solvent A. TLC of the eluate on silica gel (solvent A) resulted in two major peaks of radioactivity (Fig. 3.5A). The broad peak at R_f 0.16-0.30 contained over 50% of the eluted radioactivity; the lower R_f portion of this peak was coincident with cochromatographed zeatin 9-glucoside and the higher R_f portion with cochromatographed adenosine. The peak at R_f 0.45 contained 30% of the eluted radioactivity and cochromatographed with marker adenine. As this chromatographic system does not separate the 7- and 9-glucosides of zeatin, the eluate was chromatographed in a two-dimensional silica gel TLC system (solvent A, plate developed twice, followed by solvent B). A strip in the direction of the second dimension, which contained the cochromatographed zeatin 9-glucoside, was divided into zones for elution and determination of radioactivity (Fig.

Fig. 3.5 A. Histogram showing distribution of radioactivity after silica gel TLC (solvent A) of the zone 4 paper chromatogram eluate of a lupin seedling leaf extract. Barred lines indicate the location of cochromatographed zeatin-9-β-D-glucopyranoside (Z-9-G), adenosine (Ados) and adenine (Ad).

B. Histogram showing the distribution of radioactivity in the second dimension after silica gel TLC (solvent A 2x, solvent B) of the zone 4 paper chromatogram eluate of a lupin seedling leaf extract. The zone examined is that containing cochromatographed marker zeatin-9-β-D-glucopyranoside (Z-9-G), the location of which is indicated.


Z-9-G Ados
Ad
A
30
20
10
0
CPM(%)
0
5
10
15
Z-9-G
B
40
20
10
0
CPM(%)
0
5
10
15
DISTANCE (CM)

3.5B). Approximately 41% of the eluted radioactivity was contained in the 9-glucoside zone and a slightly greater amount in the zone (R_f 0.34) immediately behind. Synthetic zeatin 7-glucopyranoside has recently become available and was found to chromatograph at a slightly lower R_f than the 9-glucopyranoside in solvent B. Hence, from chromatographic data, the major metabolites present in the zone 4 eluate were zeatin 7- and 9-glucosides, adenine and adenosine.

Zone 5. On the basis of known R_f values (Parker and Letham, 1973), this zone could contain zeatin and zeatin riboside. TLC of this eluate on silica gel (solvent A), a system known to separate zeatin from its riboside, showed that approximately 65% of the eluted radioactivity co-chromatographed with authentic zeatin and 14% with zeatin riboside. However, this system did not distinguish between zeatin, cis-zeatin or dihydrozeatin, nor did it separate the corresponding ribosides. In solvent system G, zeatin and dihydrozeatin essentially run together but are separated adequately from cis-zeatin. A similar separation applies to the corresponding ribosides (see Table 3.1). When zone 5 eluate was chromatographed in this system the majority of the radioactivity chromatographed in the zeatin/dihydrozeatin zone; if cis-zeatin was present at all it was only a very minor metabolite. Zeatin is oxidised by dilute potassium permanganate solutions whereas dihydrozeatin (saturated N_6 side chain) is not. Permanganate oxidation of the eluate followed by TLC (solvent A) resulted in a peak of radioactivity which cochromatographed

with dihydrozeatin suggesting the presence of this metabolite. Hence the eluate was examined by the TLC system (CAMAG silica gel, solvent H) which separates zeatin, dihydrozeatin, zeatin riboside and dihydrozeatin riboside (see Table 3.1). The distribution of radioactivity over such a chromatogram is illustrated in Fig. 3.6A, the percentage of eluted radioactivity contributed by the dihydrozeatin, zeatin and zeatin riboside zones being 22.0, 33.5 and 11.5 respectively. Satisfactory separation of zeatin riboside and dihydrozeatin riboside was not obtained on this particular chromatogram due to the presence of interfering material. Development of such a chromatogram in the second dimension (solvent A) and assessment of the radioactivity in each of the marker compounds resulted in the profiles seen in Figs. 3.6B, C, D. After permanganate oxidation, the radioactivity attributed to zeatin exhibited the chromatographic behaviour of adenine; however the radioactivity which cochromatographed with dihydrozeatin was unaffected by this treatment. Thus from chromatographic evidence the major labelled components of this paper chromatogram zone eluate were zeatin, dihydrozeatin, zeatin riboside and/or dihydrozeatin riboside. A summary of the data obtained from these studies of the metabolites present in the leaf extract is presented in Table 3.2.

3.3.3. Chromatographic characterization of metabolites in the xylem sap and roots

i) Sap. Xylem sap was collected from 17-day-old

Fig. 3.6 A. Histogram showing the distribution of radioactivity after CAMAG silica gel TLC (solvent H) of the zone 5 paper chromatogram eluate of a lupin seedling extract. The location of the cochromatographed markers dihydrozeatin (diHZ), zeatin (Z) and zeatin riboside (ZR) is indicated.

B, C, D. Histograms showing the distribution of radioactivity over similar chromatograms after development in the second dimension (solvent A). The locations of the markers are indicated.

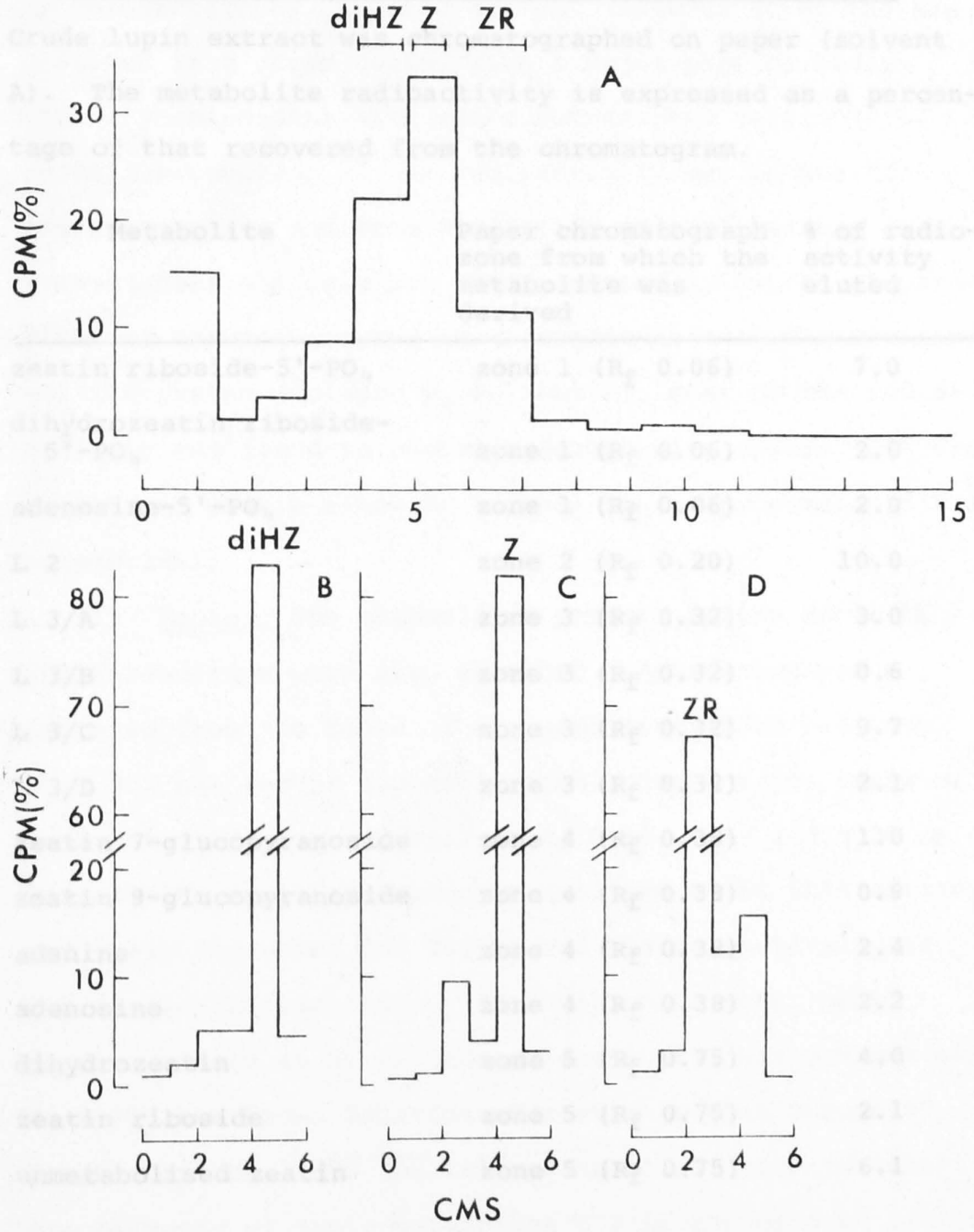

diHZ
Z
ZR
A
CPM(%)
30
20
10
0
0
5
10
15
diHZ
Z
B
C
D
ZR
80
70
60
20
10
0
0 2 4 6
0 2 4 6
0 2 4 6
CMS

Table 3.2.

Radioactivity attributable to the lupin metabolites.

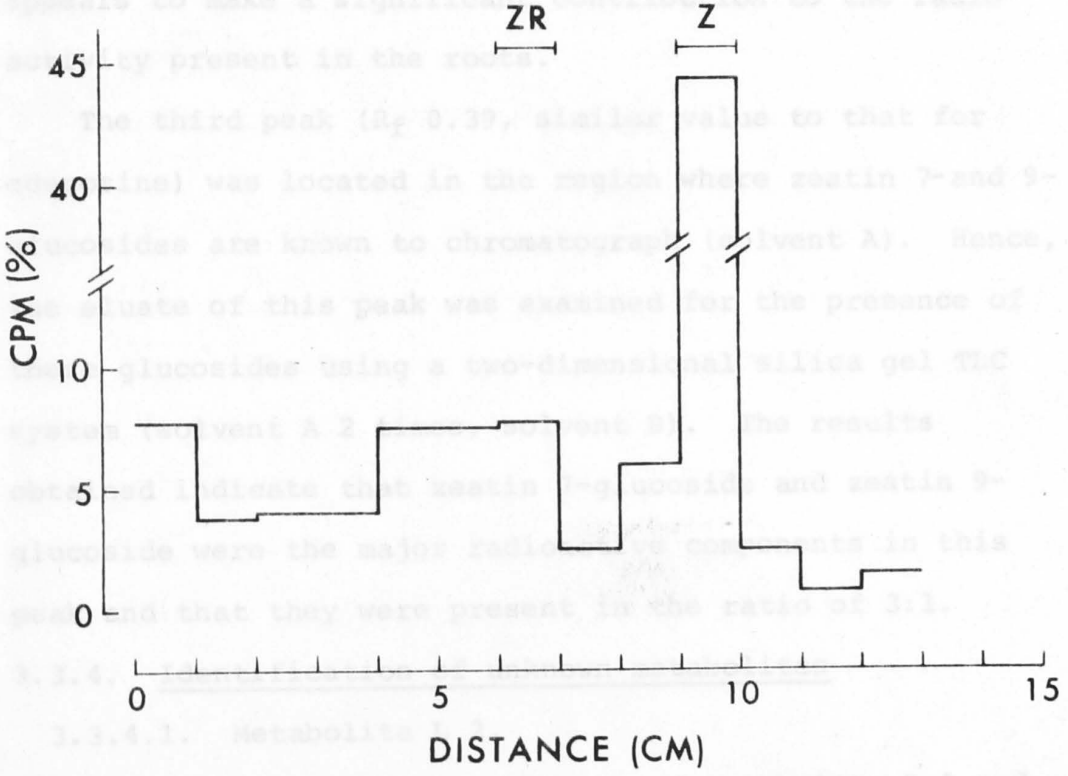
Crude lupin extract was chromatographed on paper (solvent A). The metabolite radioactivity is expressed as a percentage of that recovered from the chromatogram.

Metabolite	Paper chromatograph zone from which the metabolite was derived	% of radioactivity eluted
zeatin riboside-5'-PO_4	zone 1 (R_f 0.06)	7.0
dihydrozeatin riboside-5'-PO_4	zone 1 (R_f 0.06)	2.0
adenosine-5'-PO_4	zone 1 (R_f 0.06)	2.0
L 2	zone 2 (R_f 0.20)	10.0
L 3/A	zone 3 (R_f 0.32)	3.0
L 3/B	zone 3 (R_f 0.32)	0.6
L 3/C	zone 3 (R_f 0.32)	9.7
L 3/D	zone 3 (R_f 0.32)	2.1
zeatin 7-glucopyranoside	zone 4 (R_f 0.38)	1.0
zeatin 9-glucopyranoside	zone 4 (R_f 0.38)	0.9
adenine	zone 4 (R_f 0.38)	2.4
adenosine	zone 4 (R_f 0.38)	2.2
dihydrozeatin	zone 5 (R_f 0.75)	4.0
zeatin riboside	zone 5 (R_f 0.75)	2.1
unmetabolised zeatin	zone 5 (R_f 0.75)	6.1

seedlings (see 3.2.2.) which had been watered with [G-^{3}H]-zeatin and the metabolites present in the sap were examined by chromatography. Silica gel TLC (solvent A) of the sap resulted in a chromatogram with a major peak of radioactivity coincident with cochromatographed zeatin (Fig. 3.7); a small proportion of the radioactivity may be due to zeatin riboside and some nucleotide metabolites may be present near the origin. When a second xylem sap preparation was chromatographed in a two-dimensional CAMAG silica gel TLC system (solvent H, solvent A), most of the radioactivity was found to cochromatograph with zeatin, dihydrozeatin and zeatin riboside markers in the proportion 5:2:1 respectively.

ii) Roots. The metabolites present in the roots of lupin seedlings were also examined. An extract was prepared from the roots of the seedlings which had been used for the second sap collection. Three major peaks of radioactivity were detected at R_f 0.10, 0.17 and 0.39 on a preparative paper chromatogram (solvent A) of this extract and these accounted for 24, 11 and 11% respectively, of the eluted radioactivity. In addition, a minor peak (approximately 4% of eluted radioactivity) was present at R_f 0.75, the known location of zeatin in this chromatographic system. HVE (buffer system A) was used to detect the presence of lupin metabolite L 2 in the eluates of the peaks with R_f values of 0.10 and 0.17. This procedure showed that the metabolites present in the peak at R_f 0.10 were different from L 2. However, approximately half of

Fig. 3.7. Histogram showing the distribution of radioactivity after silica gel TLC (solvent A) of a lupin seedling sap extract. The locations of the cochromatographed markers zeatin riboside (ZR) and zeatin (Z) are indicated.


ZR
Z
45
40
10
5
0
CPM (%)
0
5
10
15
DISTANCE (CM)

the radioactivity present in the eluate from the peak at R_f 0.17 co-electrophoresed with authentic L 2. In addition, treatment of this eluate with β-glucosidase caused no change in the R_f of the radioactivity present (silica gel TLC; solvent A). Metabolite L 3/C, but not L 2, is known to be hydrolyzed by β-glucosidase. Hence L 2, but not L 3/C, appears to make a significant contribution to the radioactivity present in the roots.

The third peak (R_f 0.39, similar value to that for adenosine) was located in the region where zeatin 7- and 9-glucosides are known to chromatograph (solvent A). Hence, the eluate of this peak was examined for the presence of these glucosides using a two-dimensional silica gel TLC system (solvent A 2 times, solvent B). The results obtained indicate that zeatin 7-glucoside and zeatin 9-glucoside were the major radioactive components in this peak and that they were present in the ratio of 3:1.

3.3.4. Identification of unknown metabolites

3.3.4.1. Metabolite L 2.

The mass spectra of underivatized metabolite L 2 and of the trimethylsilyl (TMS) derivative are presented in Figs. 3.8 A and 3.9. respectively. UV spectral data is detailed in Table 3.3. Below m/e 220, the mass spectrum of underivatized L 2 is very similar to that of zeatin, the only differences being in relative peak intensities. Thus, L 2 appeared to contain an intact zeatin moiety. The UV data (Table 3.3.) is in agreement with that for an N_6,9-disubstituted adenine molecule and on this basis L 2

Fig. 3.8 A. The mass spectrum of lupin metabolite L 2.

B. The mass spectrum of synthetic β-[6-(4-hydroxy-3-methyl-but-trans-2-enylamino)purin-9-yl]alanine. The two mass spectra were obtained under identical conditions.

RELATIVE ABUNDANCE

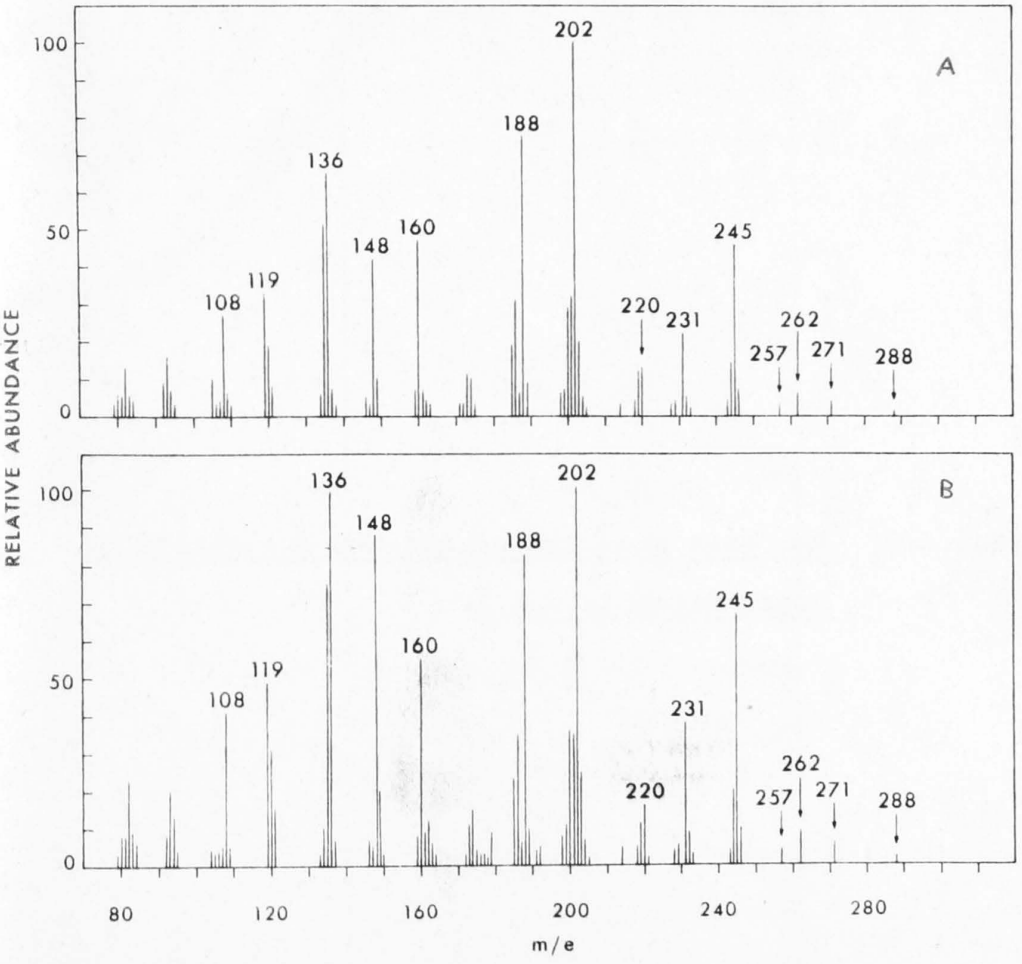

A
B
RELATIVE ABUNDANCE
100
50
0
202
188
136
148
160
245
119
108
220
231
262
257
271
288
80
120
160
200
240
280
m/e

Fig. 3.9. The mass spectrum of the TMS derivative of lupin metabolite L 2.

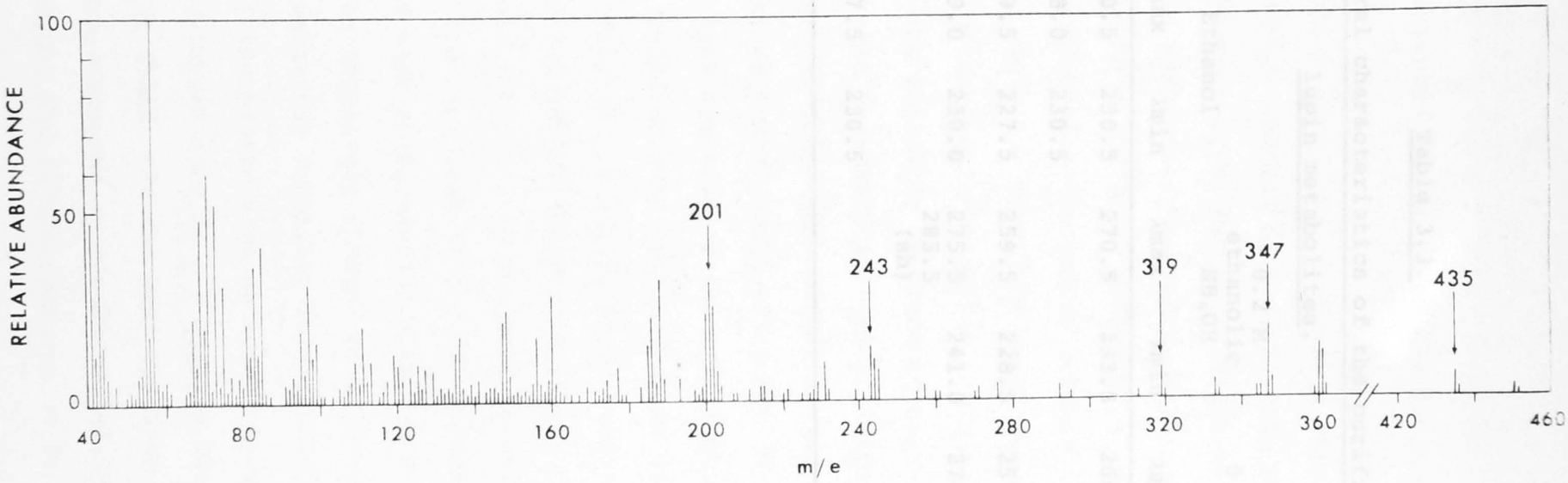

RELATIVE ABUNDANCE
100
50
0
201
243
319
347
435
40
80
120
160
200
240
280
320
360
420
460
m/e

Table 3.3.

UV spectral characteristics of the purified lupin metabolites.

Metabolite	Ethanol		0.2 M ethanolic NH_4OH		0.1 M acetic acid	
	λmax	λmin	λmax	λmin	λmax	λmin
Lupin L 2	270.5	230.5	270.5	233.5	266.5	231.5
Lupin L 3/A	268.0	230.5				
Lupin L 3/B	259.5	227.5	259.5	228.5	257.5	227.0
Lupin L 3/C	269.0	230.0	275.5 285.5 (sh)	241.0	274.0	233.5
Lupin L 3/D	267.5	230.5				

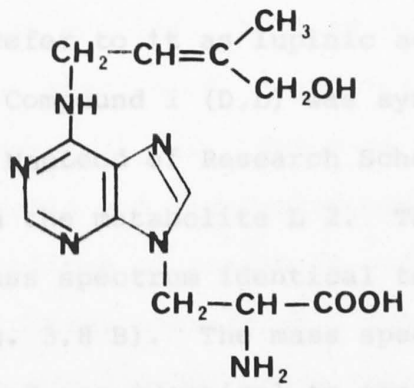
could be a 9-substituted zeatin or an O,9-disubstituted zeatin.

The mass spectrum of the TMS derivative (Fig. 3.9.) shows a molecular ion at m/e 450 and high resolution measurements established the formula $C_{19}H_{34}N_6O_3Si_2$. Since two TMS groups are present in the derivative, the molecular formula of L 2 is $C_{13}H_{18}N_6O_3$ with MW = 306 (c.f. zeatin $C_{10}H_{13}N_5O$, MW = 219). Hence, the molecular ion is not evident in the spectrum of underivatized L 2. Also, the substituent group or groups attached to the zeatin moiety in L 2 must contain two oxygen atoms and the following evidence strongly suggests that they are present in a carboxyl group. Firstly, an intense peak at m/e 44 (CO_2) was evident in the mass spectrum of underivatized L 2 although off scale in the figure. Secondly, in the mass spectrum of the underivatized compound, there are peaks derived by elimination of CO_2 at 262 (M^+-CO_2), 245 (M^+-CO_2-OH; $C_{12}H_{17}N_6$ from accurate mass measurements) and 231 ($M^+-CO_2-CH_2OH$; $C_{11}H_{15}N_6$ from accurate mass measurements). Thirdly, in the mass spectrum of the TMS derivative there is a peak at 333 (M^+-COOTMS; $C_{15}H_{25}N_6$-OSi from accurate mass measurements). Fourthly, at pH 10.2 (buffer A) L 2 was negatively charged as evidenced by its electrophoretic behaviour (mobility relative to AMP = 0.44) thus establishing the presence of an acidic group.

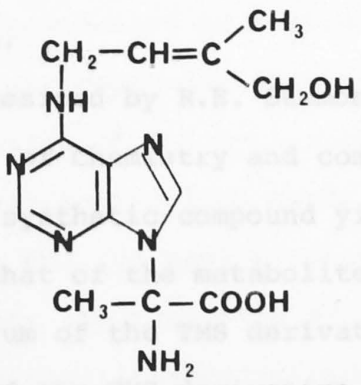
The remaining oxygen atom in L 2 must be the oxygen of the zeatin side chain. In the mass spectrum of underivatized L 2, there are peaks at m/e 257 ($M^+-H_2O-CH_2OH$) and m/e 231 ($M^+-CO_2-CH_2OH$) and in the spectrum of TMS-L 2 there

is a prominent peak at m/e 347 (M^+-CH_2OTMS). The formation of these ions involving elimination of -CH_2OH or -CH_2OTMS establishes that the oxygen of the side chain does not carry a substituent group. Hence, L 2 is a 9-substituted zeatin, the substituent being $C_3H_6NO_2$ which contains a carboxylic acid group.

L 2 reacted with ninhydrin (purple spot on TLC when sprayed and heated to 80-100°C) establishing the presence of a primary or secondary amino group. Since L 2 was degraded by L-amino acid oxidase, the metabolite contained an L-α-amino acid moiety. When the evidence presented above is considered as a whole, L 2 could have one of only two structures which are:

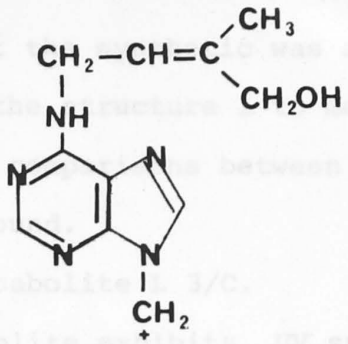


I



II

In the mass spectrum of underivatized L 2, a peak is evident at m/e 232. A high resolution spectrum established that this is due to $C_{10}C^{13}H_{15}N_6$ (3.5% of base peak) and $C_{11}H_{14}N_5O$ (3.6% of base peak). The latter is the fragment ion III derived by elimination of -$CH(NH_2)$-COOH.



III

Compound I would readily yield III but compound II would not fragment to give III. Hence, L 2 was assigned the structure I, i.e. L-β-[6-(4-hydroxy-3-methylbut-trans-2-enylamino)purin-9-yl]alanine. Since this compound was first detected in a species of lupin, it may be convenient to refer to it as lupinic acid.

Compound I (D,L) was synthesized by R.E. Summons and J.K. MacLeod of Research School of Chemistry and compared with the metabolite L 2. The synthetic compound yielded a mass spectrum identical to that of the metabolite L 2 (Fig. 3.8 B). The mass spectrum of the TMS derivative of L 2 was identical to that of the TMS derivative of synthetic I. When compared by silica gel TLC (solvents A, B and E) and by DEAE cellulose TLC (solvent 0.01 M Tris, pH 9.0), the metabolite L 2 and synthetic I were found to cochromatograph. Also, the metabolite and the synthetic compound were found to coelectrophorese in the buffers A, E, G and H. However, the synthetic compound was not completely degraded by the enzyme L-amino acid oxidase,

confirming that the synthetic was a D,L-mixture. Thus, the assignment of the structure I to metabolite L 2 was corroborated by the comparisons between the metabolite and the synthetic compound.

3.3.4.2. Metabolite L 3/C.

This metabolite exhibits UV spectra (Table 3.3.) characteristic of a 6-(monosubstituted amino)purine (c.f. λmax values for zeatin in ethanol, ethanolic NH_4OH and 0.1 N acetic acid: 269, 275 with shoulder at 285, and 274 nm respectively). The mass spectrum (Fig. 3.10.) shows a very weak molecular ion peak at m/e 381 and below m/e 220 the spectrum is similar to that of zeatin, indicating the presence of an intact zeatin moiety. However, a noticeable difference between the two spectra is the intensity of the peak at m/e 188; this is very pronounced in zeatin, and in the spectra of 7- and 9-β-D-glucopyranosylzeatin, but is weak in L 3/C. The mass spectra of 7-β-D-glucopyranosylzeatin and 9-β-D-glucopyranosylzeatin (the major metabolites of zeatin in radish and Zea mays respectively) also differ considerably from the spectrum of L 3/C in the range m/e 220-381 where there are noticeable differences in peak intensity. The spectrum of L 3/C shows a weak peak at m/e 248 (B+ 30 peak, characteristic of purine glycosides).

Metabolite L 3/C was rapidly hydrolysed by β-glucosidase, but not by α-glucosidase, to yield a compound which was chromatographically indistinguishable from zeatin; hydrolysis with a polystyrene sulphonic acid resin (H^+

Fig. 3.10. The mass spectrum of the lupin metabolite L 3/C.

L 3/C.

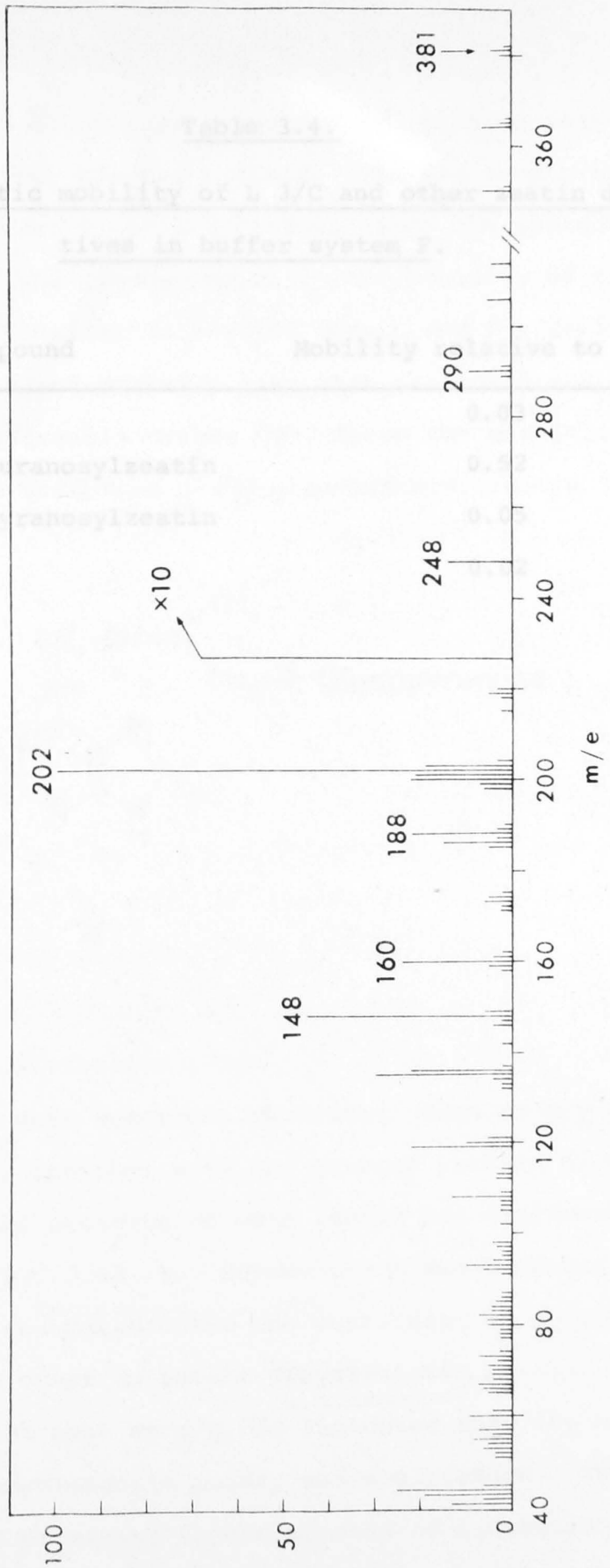
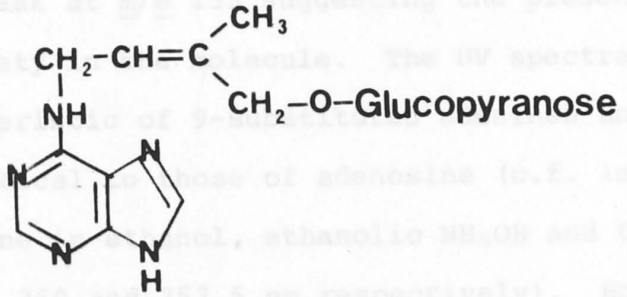

RELATIVE ABUNDANCE
100
50
0
40
80
120
160
200
240
280
360
m/e
202
188
160
148
248
290
381
×10

Table 3.4.

Electrophoretic mobility of L 3/C and other zeatin derivatives in buffer system F.

Compound	Mobility relative to AMP
L 3/C	0.03
9-β-D-glucofuranosylzeatin	0.52
9-β-D-glucopyranosylzeatin	0.05
zeatin	0.02

form) yielded glucose as evidenced by reaction with the specific enzyme, glucose oxidase. The electrophoretic mobility (buffer F) of L 3/C established the pyranoside structure of the glucose moiety; the mobility of L 3/C was almost identical to that of zeatin and 9-β-D-glucopyranosylzeatin and markedly less than that of 9-β-D-glucofuranosylzeatin (Table 3.4). Hence the metabolite was assigned the structure O-β-D-glucopyranosylzeatin (IV).



IV

3.3.4.3. Metabolite L 3/A.

In this mass spectrum, prominent peaks at m/e 162, 204, 160 and 202, together with the general pattern of peaks, indicated the presence of both zeatin and dihydrozeatin moieties (Fig. 3.12 .). Hence, L 3/A was probably a mixture of two metabolites, one containing an intact zeatin moiety, the other an intact dihydrozeatin moiety. However, the prominent peak at m/e 250 indicated that the metabolite with the dihydrozeatin moiety was a glycoside. This peak (B + 30) is prominent in the spectra of synthetic 9-β-D-

ribofuranosyldihydrozeatin and 9-β-D-glucopyranosyldihydrozeatin. Due to the small amount of sample available, a UV spectrum was obtained in neutral ethanol only and this data is insufficient to reach conclusions regarding the site of substitution in the zeatin and dihydrozeatin moieties. A mass spectrum of the TMS derivative of L 3/A did not yield additional structural information.

3.3.4.4. Metabolite L 3/B.

The mass spectrum of L 3/B (Fig. 3.13) exhibited a prominent peak at m/e 135 suggesting the presence of an adenine moiety in the molecule. The UV spectra (Table 3.2) are characteristic of 9-substituted adenines and are almost identical to those of adenosine (c.f. λmax values for adenosine in ethanol, ethanolic NH_4OH and 0.1 M acetic acid: 260, 260 and 257.5 nm respectively). However, TLC studies clearly established that L 3/B was neither adenosine nor deoxyadenosine, the R_f values for L 3/B, adenosine and deoxyadenosine on silica gel in solvent B being 0.22, 0.41 and 0.40 respectively. Hence L 3/B appeared to be considerably more polar than these two common adenine nucleosides.

The presence of an adenine moiety in L 3/B was confirmed by hydrolysis with a sulphonic acid resin. Elution of the resin (conc. NH_4OH) yielded a UV-absorbing product with the following spectral characteristics: λmax in 90% ethanol: 261 nm, λmax in 0.2 N ethanolic NH_4OH: 268 nm with a shoulder at 278.5 nm. Adenine possesses identical characteristics. The hydrolysis product and adenine could

Fig. 3.11. The mass spectrum of the lupin metabolite L 3/D.

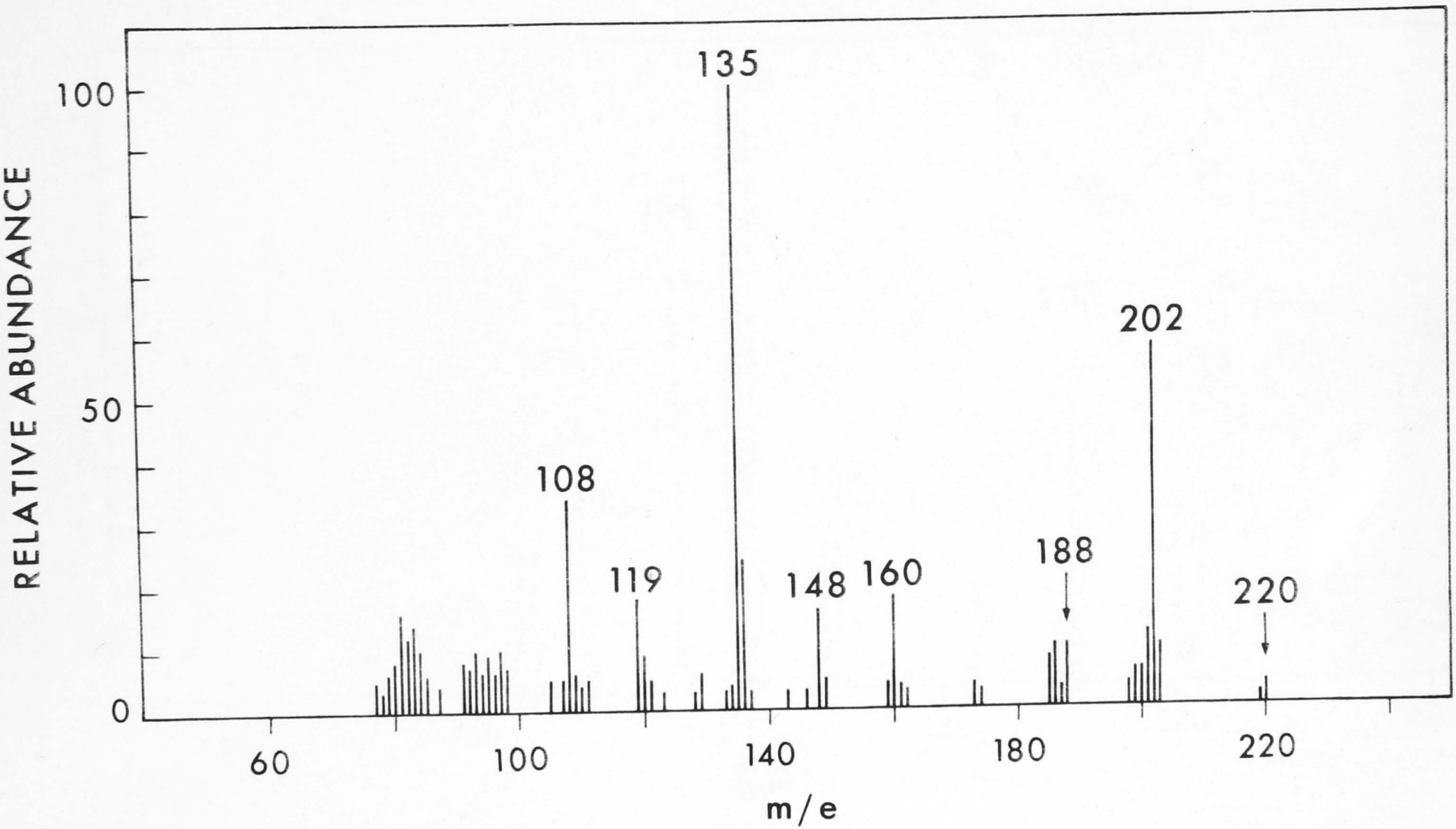

RELATIVE ABUNDANCE
100
50
0
135
202
108
119
148
160
188
220
60
100
140
180
220
m/e

Fig. 3.12. The mass spectrum of the lupin metabolite L 3/A.

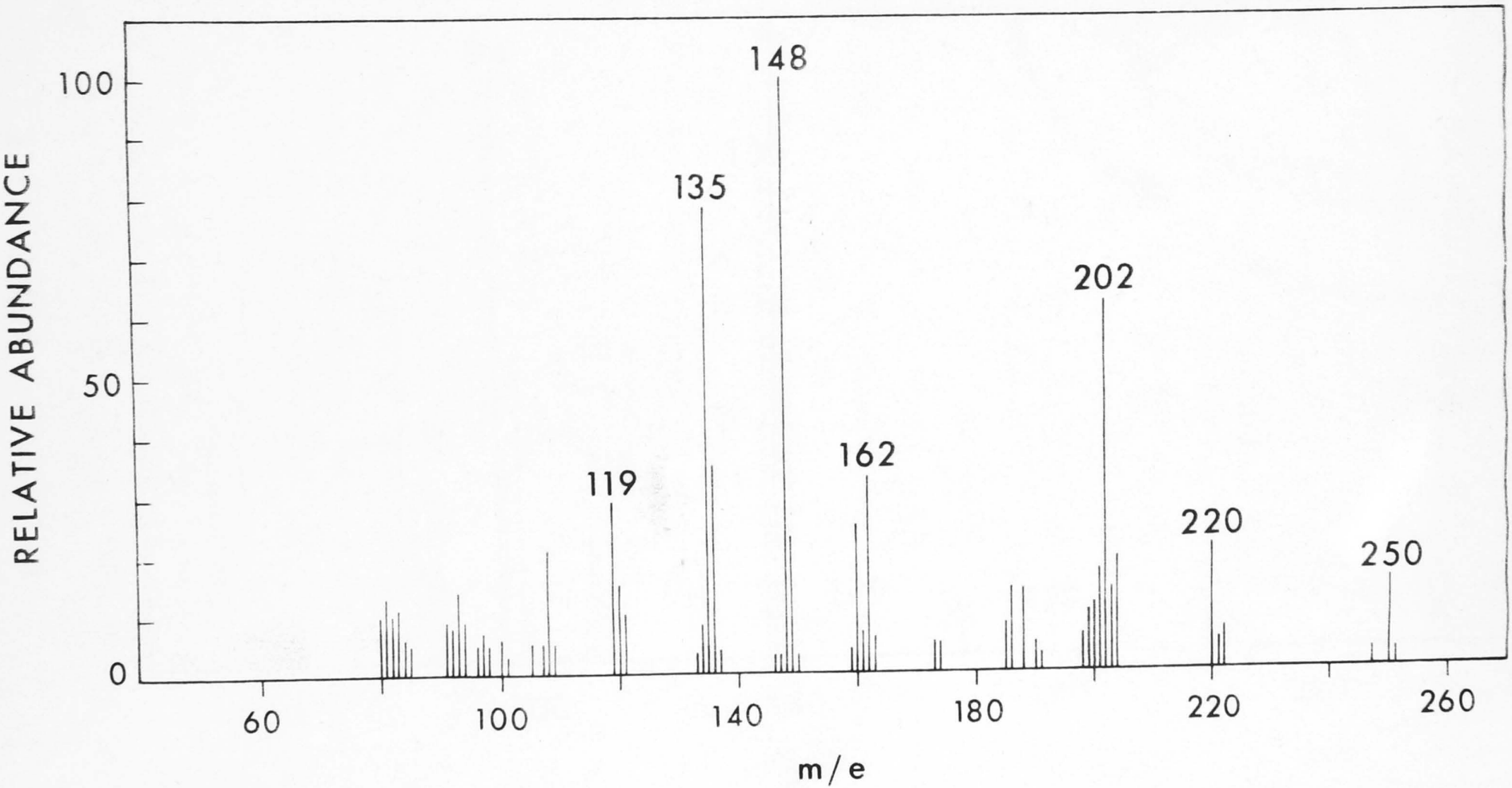
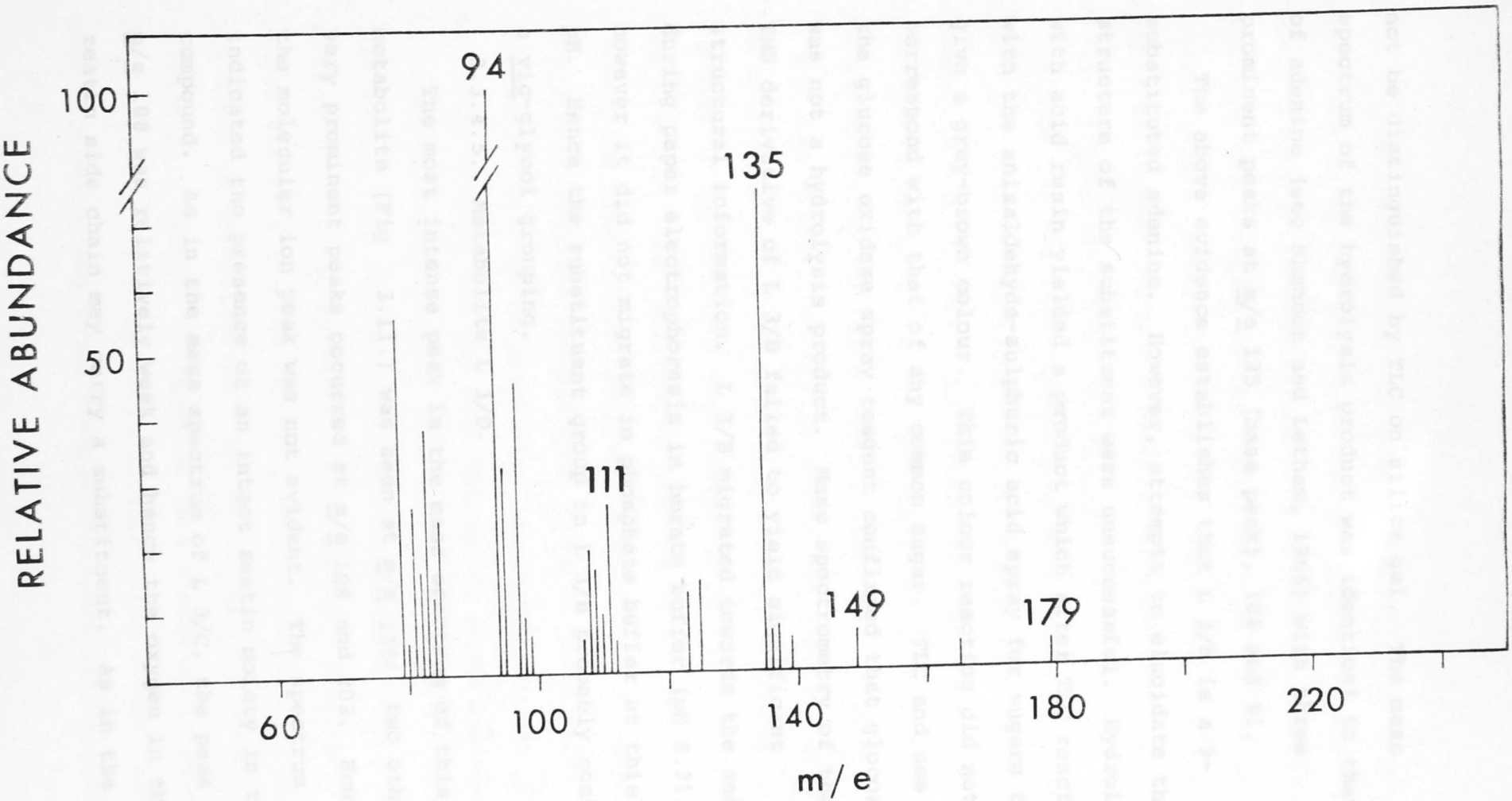

RELATIVE ABUNDANCE
100
50
0
148
135
202
162
119
220
250
60
100
140
180
220
260
m/e

Fig. 3.13. The mass spectrum of the lupin metabolite L 3/B.


RELATIVE ABUNDANCE
100
50
94
135
111
149
179
60
100
140
180
220
m/e

not be distinguished by TLC on silica gel. The mass spectrum of the hydrolysis product was identical to that of adenine (see Shannon and Letham, 1966) with three prominent peaks at m/e 135 (base peak), 108 and 81.

The above evidence establishes that L 3/B is a 9-substituted adenine. However, attempts to elucidate the structure of the substituent were unsuccessful. Hydrolysis with acid resin yielded a product which after TLC reacted with the anisaldehyde-sulphuric acid spray for sugars to give a grey-brown colour. This colour reaction did not correspond with that of any common sugar. TLC and use of the glucose oxidase spray reagent confirmed that glucose was not a hydrolysis product. Mass spectrometry of the TMS derivative of L 3/B failed to yield significant structural information. L 3/B migrated towards the anode during paper electrophoresis in borate buffer (pH 9.2); however it did not migrate in phosphate buffer at this pH. Hence the substituent group in L 3/B probably contains a vic-glycol grouping.

3.3.4.5. Metabolite L 3/D.

The most intense peak in the mass spectrum of this metabolite (Fig 3.11.) was seen at m/e 135; two other very prominent peaks occurred at m/e 108 and 202. However, the molecular ion peak was not evident. The spectrum indicated the presence of an intact zeatin moiety in the compound. As in the mass spectrum of L 3/C, the peak at m/e 188 was relatively weak and hence the oxygen in the zeatin side chain may carry a substituent. As in the case

of L 3/A, the small amount of sample available was only sufficient to obtain a UV spectrum in neutral ethanol; this did not provide sufficient data to draw conclusions regarding the location of the substituent of the zeatin moiety. A mass spectrum of the TMS derivative of L 3/D failed to provide further structural information.

3.3.5. Properties of the metabolites

All of the purified metabolites were tested for cytokinin activity in the radish cotyledon expansion bioassay. Metabolite L 2 (lupinic acid) exhibited weak activity; at a concentration of 10 μM, it induced a weight increment of 8.5 mg/cotyledon (cf. 13.0 mg/cotyledon for 10 μM zeatin in the same assay), but unlike zeatin was inactive at 2 μM. The cotyledons which had exhibited a growth response in the presence of lupinic acid were extracted in the usual way (see 2.2.1.). The resulting extract was subjected to preparative paper chromatography (solvent A) and two zones were eluted from the chromatogram. One of these zones contained a peak of radioactivity (approximately 33% of the total eluted) and was from the region where lupinic acid is known to chromatograph. HVE (buffer B) showed that more than 80% of this radioactivity electrophoresed with a very similar mobility to authentic lupinic acid. The other zone eluted was from the region where zeatin and zeatin riboside are known to chromatograph. TLC of all of this eluate (silica gel, solvent A) resulted in a single small peak of radioactivity (<0.1% of total radioactivity eluted from the paper chromatogram) which cochromatographed with authentic

Table 3.5

Cytokinin activities of the purified lupin metabolites from paper chromatogram zone 3 when tested in the same radish cotyledon expansion bioassay

Metabolite	Increment in cotyledon weight (mg/cot.)†		
	3.50 μM	1.25 μM	0.60 μM
L 3/A	-	6.9	-
L 3/B	0	-	0
L 3/C	13.6	-	10.5
L 3/D	-	8.5	-
authentic zeatin	11.5	-	11.6

†All increments represent a significant ($P<0.05$) difference between treated and control cotyledons; - indicates this concentration was not tested.

zeatin.

The metabolites L 3/A, B, C and D were also tested in the radish cotyledon expansion bioassay (see Table 3.5). Metabolite L 3/B was inactive at the concentrations tested, but L 3/A, D and C all showed activity, the last mentioned being the most active. As in the case of lupinic acid, the cotyledons from the assay of L 3/C were extracted and the extract subjected to preparative paper chromatography. TLC (silica gel, solvent A) of this zeatin zone eluate also resulted in a single weak peak of radioactivity (<0.5% of total eluted from paper chromatogram) which cochromatographed with authentic zeatin.

3.4.1. Summary

The results presented in this chapter are concerned with studies of the metabolism of [^{3}H]zeatin in lupin seedlings. The distribution of radioactivity over a paper chromatogram (solvent A) of the crude extract of the de-rooted seedlings indicated the presence of two major metabolite complexes at R_f 0.17-0.21 and 0.26-0.30; the major metabolite present in each of these complexes was purified and characterized chemically. In all, five zones (see Fig. 3.2A) were eluted from the paper chromatograms and the metabolites contained in them identified where possible, usually by means of cochromatography with authentic markers. Included among the metabolites identified in this way were zeatin riboside, zeatin 7- and 9-glucoside and zeatin nucleotide as well as adenine, adenosine and AMP. However, only zeatin, zeatin riboside and

dihydrozeatin were detected in the xylem sap of seedlings supplied with zeatin *via* the roots. The two metabolites purified and chemically characterized were L-β-[6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purin-9-yl]alanine (lupinic acid, purified from zone 2 of Fig. 3.2B) and *O*-β-D-glucopyranosylzeatin (purified from zone 3 of Fig. 3.2B). Studies of metabolites of zeatin in lupin roots revealed that lupinic acid was present, whereas *O*-β-D-glucopyranosylzeatin was not. All of the purified metabolites were tested for cytokinin activity in the radish cotyledon expansion bioassay; *O*-β-D-glucopyranosylzeatin was the most active and lupinic acid exhibited low activity at the concentrations tested. Chromatographic studies showed that the extracts of the cotyledons from the bioassay of both of these compounds contained very low levels of free zeatin.

4.1. INTRODUCTION

Information concerning cytokinin levels and identity in leaves during development and senescence is very limited. The only detailed studies available are confined to Populus X robusta (poplar) leaves. In the leaves of this species total cytokinin activity and diversity are at a maximum in young expanding leaves. As the leaves mature and senesce the number and level of the cytokinins decrease (Hewett and Wareing, 1973b). At least seven cytokinins are present in leaves of Populus X robusta. Two are probably zeatin and zeatin riboside, while a third, possibly a cytokinin glucoside, is the principal cytokinin in fully expanded lower leaves and in yellow senescent leaves (Hewett and Wareing, 1973b,c). Removal of the apex results in a marked increase in the level of this cytokinin in expanded leaves (Hewett and Wareing, 1973b). A fourth cytokinin in Populus X robusta leaves has been identified as 6-(o-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (Horgan et al., 1975). The identity of the three remaining cytokinins is not known.

Changes in cytokinin levels in buds and leaves of Populus tremula have been reported by Engelbrecht (1971). This species contains two cytokinin fractions; one was termed 'fraction Z' and contained two cytokinins with the chromatographic properties of zeatin and zeatin riboside, while the other, designated 'fraction N' appeared to be a zeatin nucleotide. The level of 'fraction Z' declines during leaf development, and in mature leaves and senescent leaves, 'fraction N' accounts for nearly all of the

cytokinin activity.

A study of zeatin metabolism in leaves of a Populus species would be a desirable complement to the studies outlined above for three reasons. Firstly, information regarding identity of unknown Populus cytokinins might result. The unidentified cytokinins in Populus could be zeatin derivatives and hence, if they were formed in sufficient amounts from exogenously supplied zeatin, their purification and identification might be achieved. Secondly, the variations in the relative levels of cytokinins in poplar leaves of different maturity may arise from differing metabolism of zeatin riboside in these leaves. Zeatin riboside is probably the dominant cytokinin present in poplar xylem sap (Hewett and Wareing, 1973a). Thirdly, comparative studies of cytokinin metabolism in leaves of differing maturity have not been reported previously for any plant species.

4.2. EXPERIMENTAL

4.2.1. Uptake of [^{3}H]zeatin and [^{3}H]zeatin riboside by poplar leaves; extraction of tissue

For studies aimed at the chromatographic characterization of zeatin metabolites, an 8 μM solution of [G-^{3}H]-zeatin (see 2.2.1.) was prepared. The petioles of detached, mature leaves of Populus alba (collected early in March) were placed in this solution and left for 120 hours in continuous fluorescent light and a gentle air current. After 24 hours most of the zeatin solution had been taken up and consequently water was supplied then and as required throughout the experiment.

On completion of the uptake, the leaves (17.0 g) were extracted with 80% methanol as described previously in section 2.2.1. After evaporation (<35°C) the extract was taken up in 50% ethanol (1.0 ml/g fresh weight) and then clarified by low speed centrifugation. The resulting supernatant was evaporated and the residue redissolved in 50% ethanol (0.25 ml/g fresh weight) for chromatography.

The metabolism of zeatin riboside in poplar leaves at varying stages of maturity was also investigated. Petioles of leaves collected in autumn were placed in a solution of [8-^{3}H]zeatin riboside (4.0 μM) and left for 100 hours in continuous fluorescent light and a gentle air current. They were then extracted as described above. The evaporated extract was taken up in 50% ethanol (1.0 ml/1.5 g fresh weight).

4.2.2. Purification of the major metabolites of zeatin

Mature leaves of Populus alba (220 g) were supplied with a 100 μM solution of unlabelled zeatin (2.0 litres) and extracted as described previously (4.2.1.). The evaporated extract was suspended in water (150 ml), brought to pH 3.0 and percolated through a column of cellulose phosphate (NH_4^+ form equilibrated to pH 3.0; 300 ml) which was then washed with water (1.8 litres at pH 3.0) and finally eluted with 0.3 M NH_4OH (2.4 litres). After evaporation the eluate was dissolved in 50% ethanol (30.0 ml).

Previously, an extract had been prepared from leaves supplied with ^{3}H-zeatin and this extract had been chromatographed on paper in solvent A (see 4.3.2.). Portion of the zone 3 eluate from this chromatogram (see Fig. 4.1 B) was mixed with the column eluate (2.5 ml) and chromatographed on a 1.0 mm silica gel thin layer (solvent A). The distribution of radioactivity over a narrow strip from this plate was determined. Two peaks of radioactivity which were coincident with UV absorbing zones at R_f 0.37 (zone 1) and R_f 0.46 (zone 2) were detected. The remainder of the column eluate was then chromatographed and the zones 1 and 2 were scraped from the plates, combined with unused 'labelled' zones from the first chromatogram and eluted exhaustively with methanol-water-acetic acid (80:20:1). The zone 1 and 2 material now contained a low level of radioactivity which served as a marker during subsequent purification steps.

A further preparative TLC step with the zone 1 and 2 eluates (0.5 mm PF_{254} silica gel; solvent B; 5 plates

each eluate) yielded single peaks of radioactivity which were coincident with discrete UV absorbing zones in each case. The relative level of radioactivity present in the two peaks was approximately 1.5 (peak from zone 1) and 1.0 (peak from zone 2) whereas the UV intensity associated with the zone 2 peak was much greater than that of the zone 1 peak. The silica gel containing the radioactive zones was scraped from the chromatograms and exhaustively eluted with methanol-water-acetic acid (80:20:1). The eluates were evaporated and redissolved in 50% ethanol (1.0 ml each) for further chromatography.

The eluate obtained by rechromatographing zone 2 on silica gel in solvent B was subjected to paper chromatography (washed paper, solvent A). This yielded a single UV absorbing component (R_f 0.37). This metabolite, P 2, was eluted with 50% ethanol for UV spectra and mass spectrometry.

Purification of the fraction derived from zone 1 was complex and is therefore outlined in a flow diagram which also indicates earlier purification steps including those leading to the purification of P 2 (Fig. 4.3). Paper chromatography of the eluate derived from zone 1 resulted in two UV absorbing zones (X = low R_f, Y = higher R_f) which were separated from each other by an intense yellow-brown zone. Both of these zones (X and Y) were eluted for further purification by TLC on silica gel layers (0.5 mm, solvent A developed twice). By this method, the zone X eluate was resolved into three UV absorbing zones (X1-3) each

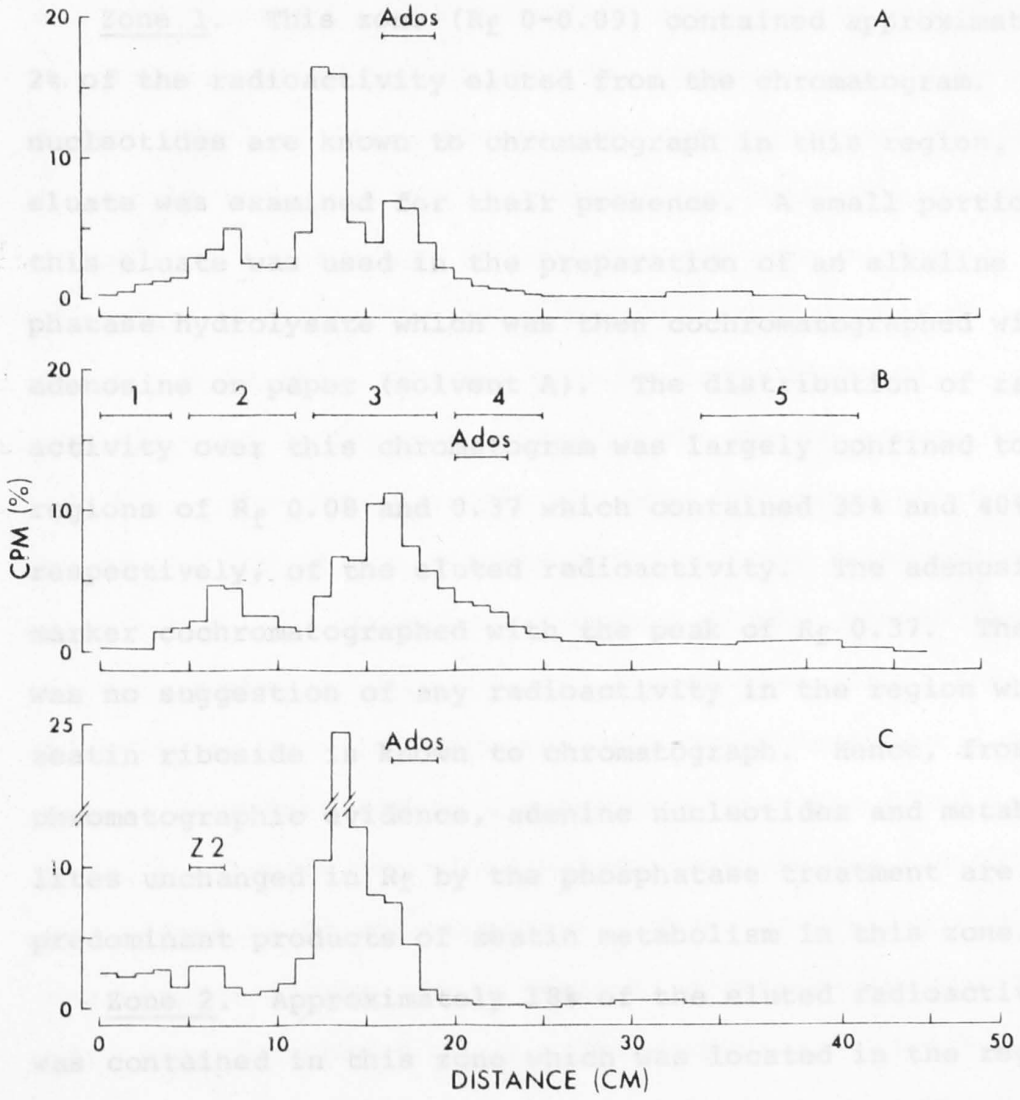
possessing a low level of radioactivity, whereas the Y eluate was separated into five UV absorbing zones (Y1-5) of which three (distance from origin: 2.8, 3.7, 4.5 cm) contained a high proportion of the radioactivity. These three zones (Y2, Y3 and Y4) were exhaustively eluted (methanol-water-acetic acid, 80:20:1) evaporated and dissolved in 50% ethanol for final purification by chromatography on washed paper (solvent D). Y2 yielded a single UV absorbing zone of R_f 0.53, but Y3 and Y4 were each separated into three UV absorbing zones of R_f 0.28, 0.39, 0.46, and 0.34, 0.39, 0.47 respectively. The Y2 zone was eluted in 80% ethanol (redistilled solvents, 4.0 ml) to give metabolite Y2/1 (25 µg approx.) for UV and mass spectrometry. A narrow strip (0.5 cm wide) was taken from the middle of the Y3 and Y4 chromatograms for the determination of the distribution of radioactivity over each. The results obtained showed that the Y3 zone of R_f 0.39 and the Y4 zone of R_f 0.47 each contained almost all of the radioactivity present on each chromatogram. Thus, these zones were eluted with 80% ethanol (redistilled solvents, 5.0 ml) to yield the metabolites Y3/2 (90 µg approx.) and Y4/3 (100 µg approx.) for characterization by UV and mass spectrometry.

4.3. RESULTS

4.3.1. Paper chromatography of crude extract of leaves supplied with zeatin

The mature leaves of a clone of Populus alba were supplied with a solution of [G-^{3}H]zeatin via their petioles and an extract prepared from them (see 4.2.1.). The extract was chromatographed on paper (solvent A) and the distribution of radioactivity over the chromatogram was determined (see Fig. 4.1 A). Three prominent peaks of radioactivity were detected, at R_f 0.15, 0.28 and 0.37, which contained 10, 42 and 14% respectively of the eluted radioactivity. Cochromatographed adenosine was coincident with the peak of R_f 0.37 on this chromatogram. Comparison of Fig. 4.1 A and Fig. 3.2 A showed that the major metabolite peak of the poplar leaf extract occurred at a similar position to a major metabolite peak of a lupin leaf extract which had been chromatographed under equivalent conditions. Several unidentified zeatin metabolites were purified from this lupin extract zone and hence further investigation of the metabolites from the poplar extract zone seemed warranted. Thus, a preparative paper chromatogram (solvent A) of the crude extract was run and the distribution of radioactivity over a narrow strip was determined (Fig. 4.1 B). The profile of the histogram in Fig. 4.1 B differs slightly from that of Fig. 4.1 A, the major difference being that the minor peak coincident with adenosine in Fig. 4.1 A appears only as a shoulder in 4.1 B.

Fig. 4.1. The distribution of radioactivity over paper chromatograms (solvent A) of extracts of poplar leaves which had been supplied with [G-^{3}H]-zeatin. A: preliminary paper chromatogram, B: paper chromatogram used for elution of zones, C: paper chromatogram of extract after chromatography on a cellulose phosphate column. The barred line Ados denotes the position on the chromatogram of cochromatographed adenosine. The barred lines 1-5 of 1B denote zones eluted for further investigation. The barred line Z 2 of 1C denotes the zone eluted for comparison with authentic lupinic acid.


A
Ados
20
10
0
B
1
2
3
4
5
Ados
CPM (%)
C
Ados
Z 2
25
0
10
20
30
40
50
DISTANCE (CM)

4.3.2. Metabolites in paper chromatogram zones

The zones 1-5 depicted on the histogram (Fig.4.1 B) were eluted for investigation of the metabolites present.

Zone 1. This zone (R_f 0-0.09) contained approximately 2% of the radioactivity eluted from the chromatogram. As nucleotides are known to chromatograph in this region, the eluate was examined for their presence. A small portion of this eluate was used in the preparation of an alkaline phosphatase hydrolysate which was then cochromatographed with adenosine on paper (solvent A). The distribution of radioactivity over this chromatogram was largely confined to two regions of R_f 0.08 and 0.37 which contained 35% and 40%, respectively, of the eluted radioactivity. The adenosine marker cochromatographed with the peak of R_f 0.37. There was no suggestion of any radioactivity in the region where zeatin riboside is known to chromatograph. Hence, from chromatographic evidence, adenine nucleotides and metabolites unchanged in R_f by the phosphatase treatment are the predominant products of zeatin metabolism in this zone.

Zone 2. Approximately 18% of the eluted radioactivity was contained in this zone which was located in the region R_f 0.13-0.23. This is the region in which lupin metabolite L 2 (lupinic acid) chromatographed under identical conditions (see 3.3.2. zone 2). An aliquot of the zone 2 eluate was subjected to silica gel TLC (solvent A, developed twice) and the distribution of radioactivity over the chromatogram was determined. A single, major peak of radioactivity which contained >70% of the eluted radio-

activity was located in a zone 1.0-3.0 cm from the origin. Lupinic acid is known to travel further in this chromatographic system. To prove conclusively that lupinic acid was not present in this zone, the behaviour of the eluate was studied using HVE (buffer A). However, the crude eluate streaked badly during electrophoresis and therefore, to remove the interfering material the remainder of the crude leaf extract was chromatographed on a cellulose phosphate column (NH_4^+ form, equilibrated to pH 3.0) and the 0.3 M NH_4OH eluate collected. Under these circumstances, lupinic acid is known to occur in the column eluate (see 3.3.2.). The column eluate was then chromatographed on paper and the eluate corresponding to zone 2 was prepared (see Fig. 4.1C). Co-electrophoresis of this eluate (buffer A) with authentic lupinic acid showed that <2% of the radioactivity on the electrophoretogram could have been lupinic acid. More than 50% of the radioactivity was found in a broad peak which travelled mid-way between lupinic acid and adenosine 5'-monophosphate. Thus, if any lupinic acid was present in the zone 2 metabolite complex, it was a very minor component of it. The compounds 7- and 9-β-D-glucopyranosyladenine (prepared synthetically) were found to chromatograph at a similar R_f to the zone 2 complex on silica gel (solvent A). The 'purified' zone 2 eluate was cochromatographed with these two compounds on a cellulose thin layer in solvent B. Under these conditions, >73% of the eluted radioactivity chromatographed in a peak immediately in front of adenine 9-glucopyranoside (R_f 0.53)

while adenine 7-glucopyranoside travelled close behind at R_f 0.47. Together, these two compounds could only account for 10% of the eluted radioactivity. In summary, the chromatographic and electrophoretic evidence presented suggests that adenine 7- and 9-glucopyranosides and lupinic acid are not present or are minor metabolites, in the zone 2 eluate. The major metabolite(s) were not identified.

Zone 3. This zone (R_f 0.28-0.40) contained the major peak of radioactivity eluted from the paper chromatogram (>50%) and it chromatographed in the region slightly behind adenosine (Fig. 4.1B). Silica gel TLC of the eluate (solvent A, developed twice) separated the radioactivity into three distinct peaks which contained, in order of increasing distance from the origin, 62, 12 and 4% of the total eluted (Fig. 4.2.). The peak containing 12% of the radioactivity cochromatographed with hypoxanthine and that containing 4% with adenine. However, the major peak was located much closer to the origin. Its position corresponded to that of the major peak of radioactivity present in the equivalent paper chromatogram zone eluate (zone 3) of a lupin leaf extract (see 3.3.2. Zone 3 and Fig. 3.2 A). Further, the poplar complex exhibited similar behaviour to the lupin complex on a cellulose phosphate ion exchange column, being retained when washed with water at pH 3.0 and eluted by 0.3 M NH_4OH (Fig. 4.1C). The purification and identification of the major metabolites in this zone was undertaken as described elsewhere (4.2.2. and Fig. 4.3.).

Zone 4. This zone, which encompassed cochromatographed

Fig. 4.2. Histogram showing the distribution of radioactivity after silica gel TLC (solvent A , developed twice) of the zone 3 paper chromatogram eluate (see Fig. 4.1B) of a poplar leaf extract. The barred lines indicate the location of cochromatographed adenine (Ad) and hypoxanthine (Hypo).

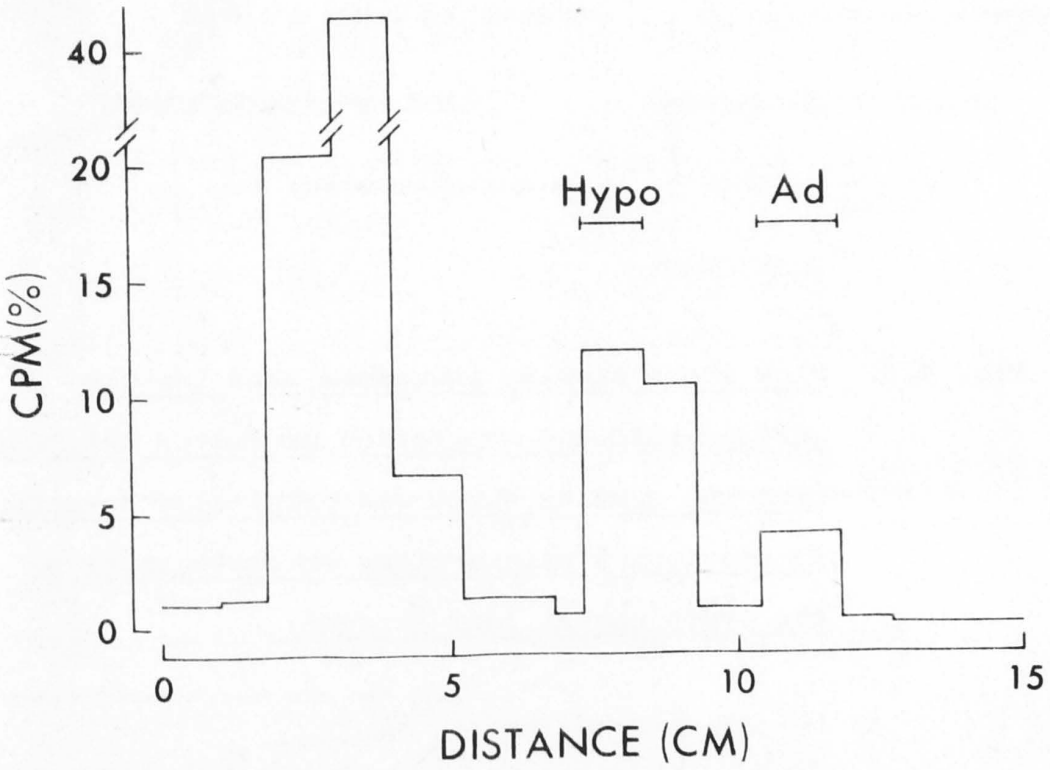
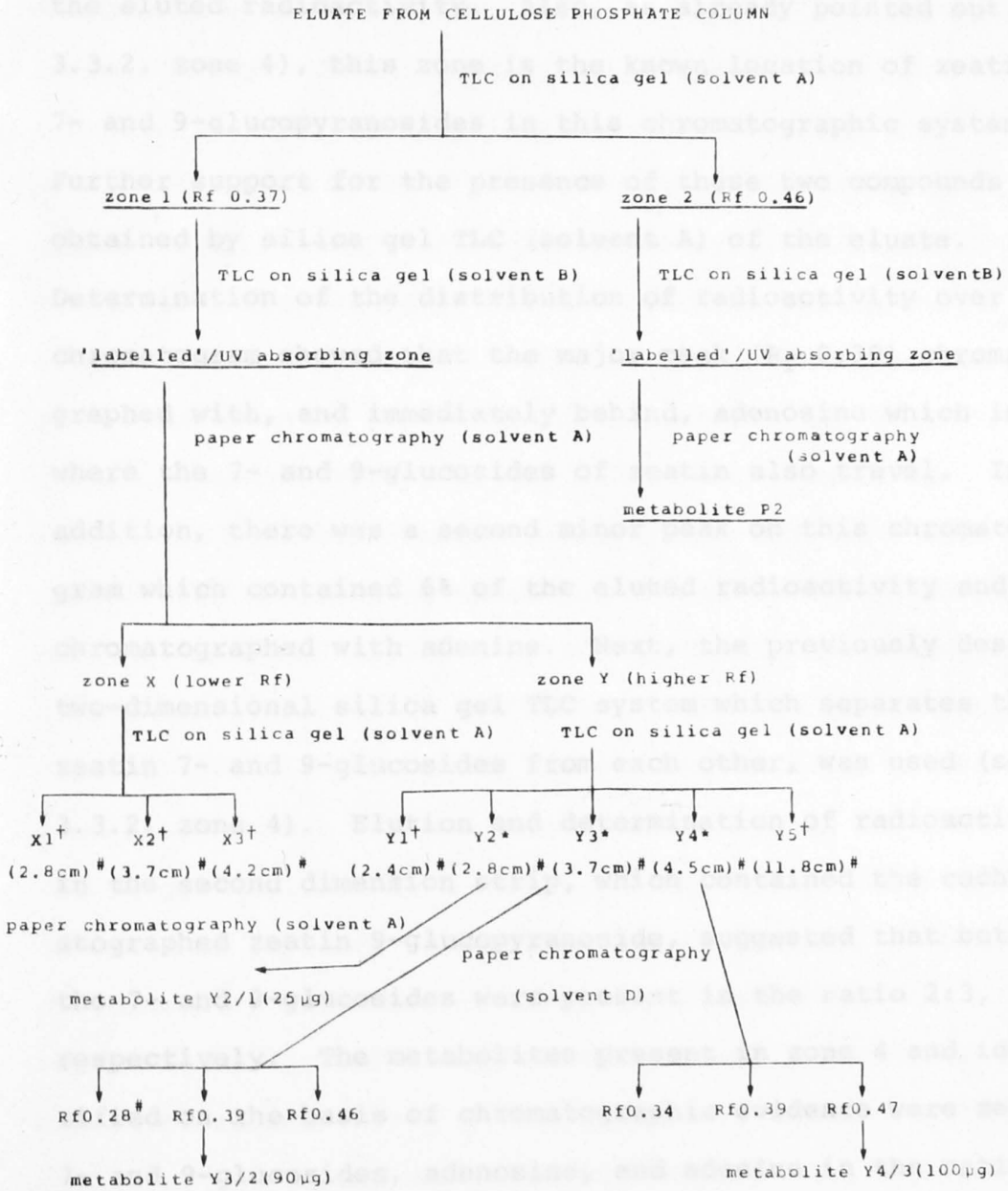

40
20
15
10
5
0
CPM(%)
Hypo
Ad
0
5
10
15
DISTANCE (CM)

Fig. 4.3. Flow sheet showing procedure used for the purification and separation of zeatin metabolites from the complex which was originally detected in the zone 3 eluate after chromatography of the crude poplar leaf extract.



†.: low or negligible radioactivity

* : pronounced radioactivity

\# : distance travelled from the origin.

adenosine (see Fig. 4.1B), contained approximately 11% of the eluted radioactivity. Also, as already pointed out (see 3.3.2. zone 4), this zone is the known location of zeatin 7- and 9-glucopyranosides in this chromatographic system. Further support for the presence of these two compounds was obtained by silica gel TLC (solvent A) of the eluate. Determination of the distribution of radioactivity over this chromatogram showed that the major peak (R_f 0.30) chromatographed with, and immediately behind, adenosine which is where the 7- and 9-glucosides of zeatin also travel. In addition, there was a second minor peak on this chromatogram which contained 6% of the eluted radioactivity and cochromatographed with adenine. Next, the previously described two-dimensional silica gel TLC system which separates the zeatin 7- and 9-glucosides from each other, was used (see 3.3.2. zone 4). Elution and determination of radioactivity in the second dimension strip, which contained the cochromatographed zeatin 9-glucopyranoside, suggested that both the 7- and 9-glucosides were present in the ratio 2:3, respectively. The metabolites present in zone 4 and identified on the basis of chromatographic evidence were zeatin 7- and 9-glucosides, adenosine, and adenine in the ratio 3.2:4.8:3.0:1.0, respectively.

Zone 5. Only 3% of the radioactivity present on the paper chromatogram was contained in this zone which is also the known location of zeatin, dihydrozeatin and their ribosides in this chromatographic system. Silica gel TLC (solvent A) of the eluate in the presence of cochromato-

graphed zeatin and zeatin riboside showed that they were coincident with 40 and 20%, respectively, of the eluted radioactivity. This chromatographic system does not separate zeatin from dihydrozeatin. To overcome this problem, portion of the 'purified' paper chromatogram eluate (i.e. chromatographed on paper after cellulose phosphate chromatography) was subjected to TLC on an alumina layer (solvent J). This system gives a particularly good separation of zeatin and dihydrozeatin (R_f 0.25) from their respective ribosides (R_f 0.06) which remain near the origin and from other minor metabolites. The zone coincident with cochromatographed zeatin was eluted exhaustively with 80% methanol followed by 80% acetone. The evaporated eluate was dissolved in H_2O (250 µl) and extracted with n-butanol (200 µl; to remove interfering material eluted from the alumina) prior to two-dimensional TLC on CAMAG silica gel (solvent H, developed three times; solvent A) in order to separate zeatin from dihydrozeatin. Authentic dihydrozeatin was added to the butanol extract of the eluate before it was loaded onto the plate. The distribution of radioactivity in the zeatin and dihydrozeatin strips of the second dimension was examined and each was found to contain only one peak of radioactivity which was coincident with the marker compounds. In summary, the total amount of radioactivity eluted from this zone was low and on the basis of chromatographic studies it contained zeatin, dihydrozeatin and zeatin riboside in the ratio 4:1:2, respectively.

4.3.3. Identification of unknown metabolites of zone 3

4.3.3.1. Resin hydrolyses.

When hydrolysed with ZEOKARB 225 (H^+ form, see 2.2.7.), Y 2/1, Y 3/2 and Y 4/3 all yielded glucose which was identified using the specific enzyme glucose oxidase, after TLC of the hydrolysates. The elucidation of the structure of these three glucoside metabolites, and also of P 2, is outlined below.

4.3.3.2. Metabolite Y 2/1.

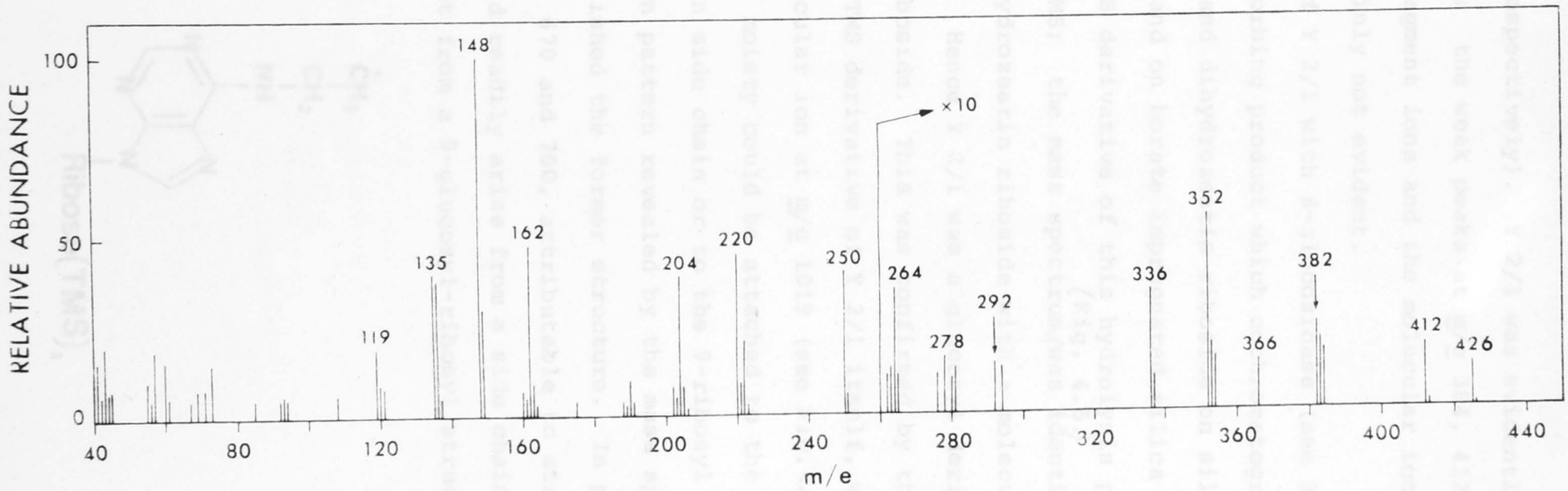
The UV spectra established that Y 2/1 was an $\underline{N}_6$,9-disubstituted adenine (see Table 4.1). The mass spectrum of the underivatized compound (Fig. 4.4.) indicated the presence of a dihydrozeatin moiety. Below $\underline{m}/\underline{e}$ 222 all fragment ions could be attributed to this structural feature; furthermore, the prominent peak at $\underline{m}/\underline{e}$ 250 could be assigned to the B+30 fragment ion which would be produced by a 9-glycoside of dihydrozeatin. Initially, it was suspected that Y 2/1 was simply the 9-glucoside of dihydrozeatin (M.W. 383) although the very weak peaks at $\underline{m}/\underline{e}$ 384, 412 and 426 were not in complete accord with this view. However these peaks, and also the peak at $\underline{m}/\underline{e}$ 383, were so weak they could have been due to impurities. 9-β-D-Glucopyranosyldihydrozeatin was therefore prepared synthetically by Dr. C. Duke (R.S.C.) for comparison with Y 2/1. The mass spectra for the two compounds, while similar, did show some significant differences in peak intensity. TLC on silica gel (solvent A) clearly established that the synthetic 9-glucoside and Y 2/1 were different (R_f values

Table 4.1

UV spectral characteristics of the purified poplar metabolites.

Metabolite	80% ethanol		0.2 M ethanolic NH_4OH		0.1 M acetic acid	
	λmax	λmin	λmax	λmin	λmax	λmin
Y2/1	267.0	230.0	267.0	-	264.0	229.5
Y3/2	269.5	229.0	274.5 (285.0 sh)	240.0	273.5	233.5
Y4/3	270.0	230.0	275.0 (285.0 sh)	240.0	273.0	234.0
P2	260.0	227.0	260.5	227.5	257.5	227.5

Fig. 4.4. The mass spectrum of poplar leaf metabolite Y 2/1.


RELATIVE ABUNDANCE
100
50
0
148
×10
352
162
135
220
204
250
264
336
382
292
119
278
366
412
426
40
80
120
160
200
240
280
320
360
400
440
m/e

0.23 and 0.17, respectively). Y 2/1 was evidently a more complex molecule; the weak peaks at m/e 384, 412 and 426 were probably fragment ions and the molecular ion of Y 2/1 was almost certainly not evident.

Hydrolysis of Y 2/1 with β-glucosidase (see 3.2.4.) yielded a UV-absorbing product which cochromatographed with zeatin riboside and dihydrozeatin riboside on silica gel (solvents A, E) and on borate impregnated silica gel (solvent I). The TMS derivative of this hydrolysis product was subjected to GC-MS; the mass spectrum (Fig. 4.5.) was identical to that of synthetic dihydrozeatin riboside with a molecular ion peak at m/e 641. Hence Y 2/1 was a glucosyl derivative of dihydrozeatin riboside. This was confirmed by the mass spectrum of the TMS derivative of Y 2/1 itself, which exhibited a molecular ion at m/e 1019 (see Fig. 4.6.).

The glucosyl moiety could be attached to the oxygen of the dihydrozeatin side chain or to the 9-ribosyl moiety. The fragmentation pattern revealed by the mass spectrum of TMS-Y 2/1 established the former structure. In particular, ions at m/e 510, 670 and 700, attributable to structures I, II and III, would readily arise from a side chain O-glucosyl structure but not from a 9-glucosyl-ribosyl structure. The

I

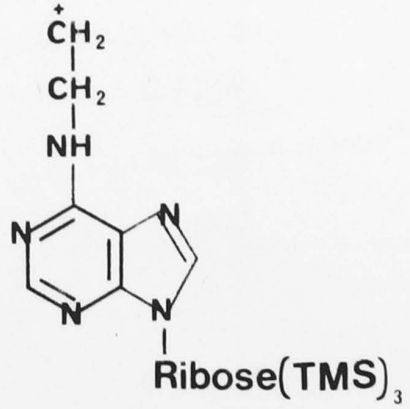
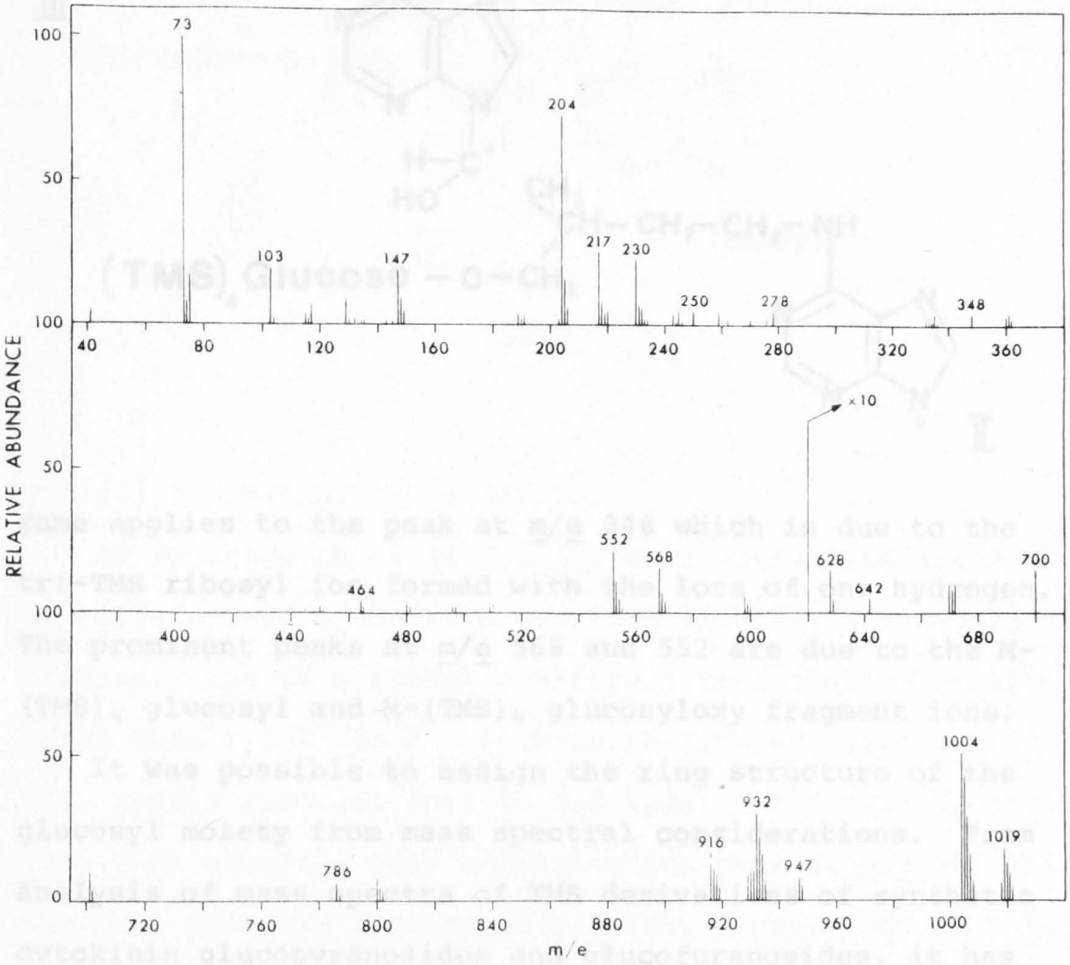
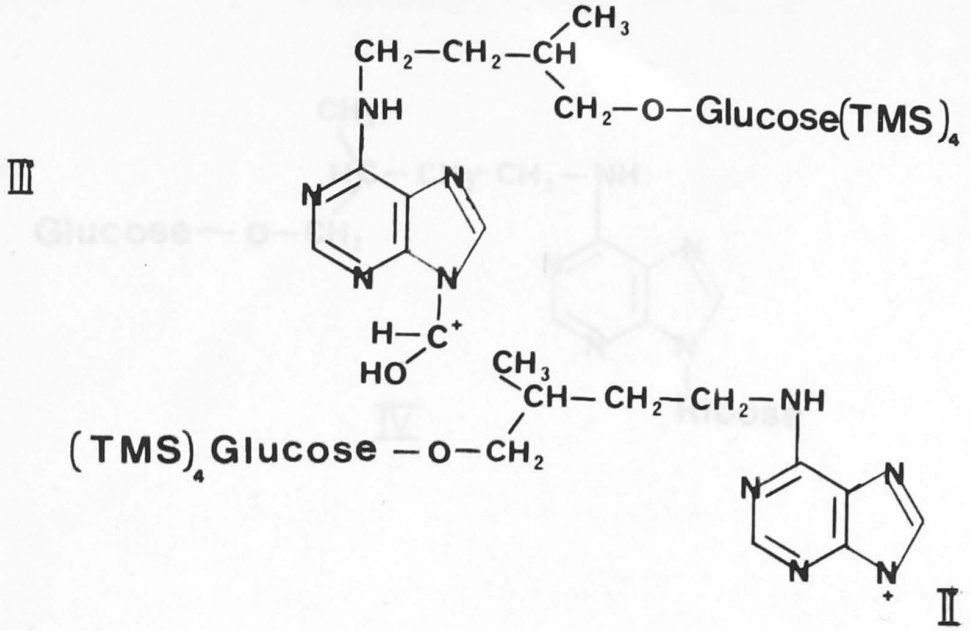


Fig. 4.5. The mass spectrum of the TMS derivative of the β-glucosidase hydrolysis product of poplar metabolite Y 2/1.

00
50
0
73
103
115
146
162
169
203
217
230
245
259
294
322
×10
348
366
80
120
160
200
240
280
320
360
50
0
393
401
408
423
462
483
498
510
538
551
626
641
400
440
480
520
560
600
640
680
m/e

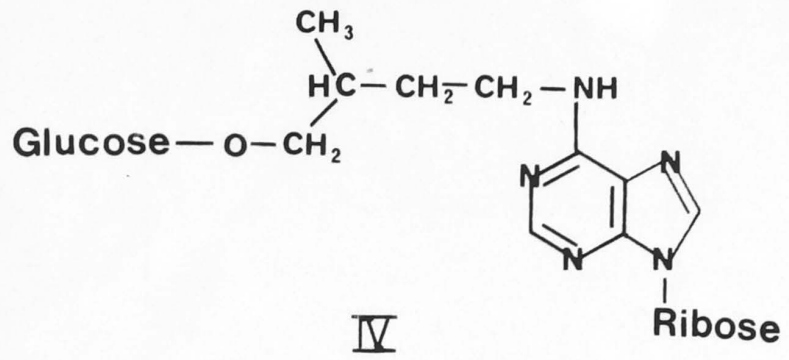
Fig. 4.6. The mass spectrum of the TMS derivative of poplar metabolite Y 2/1.


RELATIVE ABUNDANCE
m/e
73
103
147
204
217
230
250
278
348
×10
464
552
568
628
642
700
786
916
932
947
1004
1019



same applies to the peak at m/e 348 which is due to the tri-TMS ribosyl ion formed with the loss of one hydrogen. The prominent peaks at m/e 568 and 552 are due to the M-$(TMS)_4$ glucosyl and M-$(TMS)_4$ glucosyloxy fragment ions.

It was possible to assign the ring structure of the glucosyl moiety from mass spectral considerations. From analysis of mass spectra of TMS derivatives of synthetic cytokinin glucopyranosides and glucofuranosides, it has been shown very recently that the intensity of the m/e 205 peak relative to the 204 peak is low in pyranosides but high in furanosides (MacLeod et al., 1976). Hence from Fig.4.4.it is evident Y 2/1 is a glucopyranoside. It was therefore assigned structure IV i.e. 6-(4-β-D-glucopyranosyloxy-3-methylbutyl-amino)-9-β-D-ribofuranosylpurine.

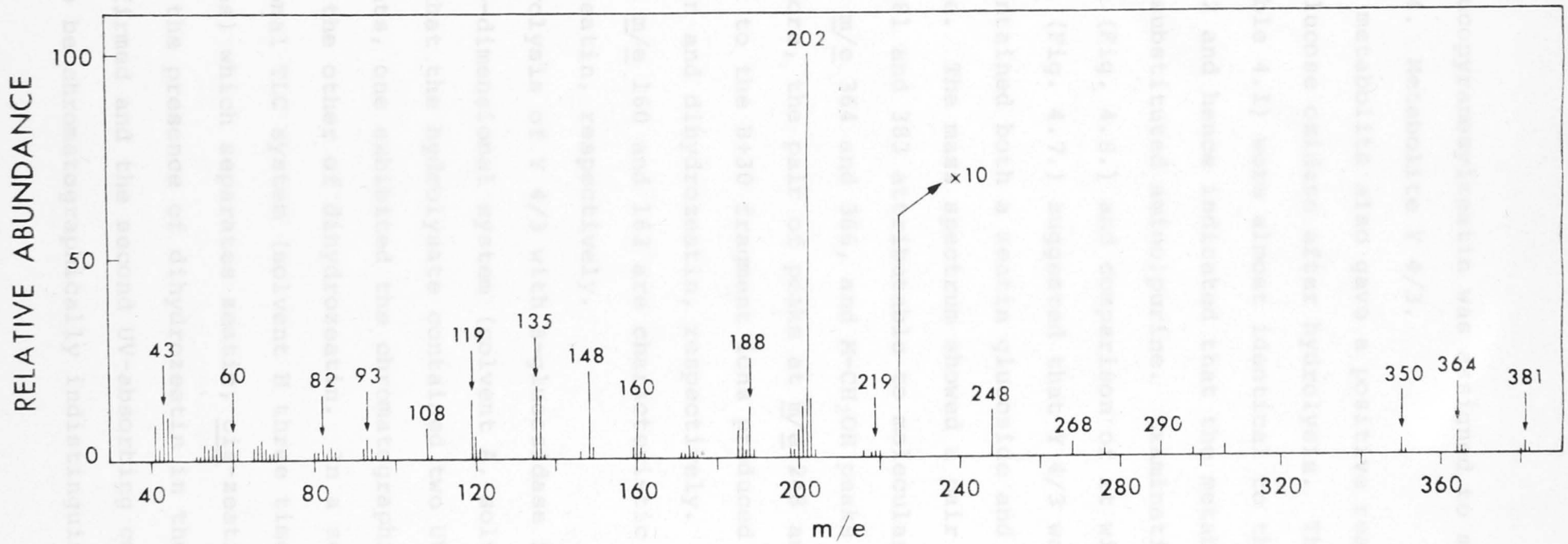


IV

4.3.3.3. Metabolite Y 3/2.

As reported above (4.3.3.1.) this metabolite after hydrolysis gave a positive reaction with the enzyme glucose oxidase. The UV spectral characteristics (Table 4.1) indicated that Y 3/2 was a 6-(monosubstituted amino)purine (cf. zeatin spectral data in 3.3.4.2.). The mass spectrum (Fig. 4.7) showed a weak molecular ion peak at m/e 381 and the base peak was located at m/e 202. Comparison of this mass spectrum with that of lupin metabolite L 3/C (Fig. 3.10.) indicated that this poplar metabolite and L 3/C were identical. This conclusion was confirmed by the identical behaviour of the two metabolites on silica gel TLC (solvent A, two times). Treatment of Y 3/2 with the enzyme β-glucosidase yielded a product which was indistinguishable from authentic zeatin by TLC in solvents A and G. The extremely low mobility of the metabolite during HVE in borate at pH 9.2 (buffer F.) indicated that the glucose moiety was in the pyranose form. Therefore, the structure

Fig. 4.7. The mass spectrum of the poplar metabolite Y 3/2.


RELATIVE ABUNDANCE
100
50
0
202
×10
43
60
82
93
108
119
135
148
160
188
219
248
268
290
350
364
381
40
80
120
160
200
240
280
320
360
m/e

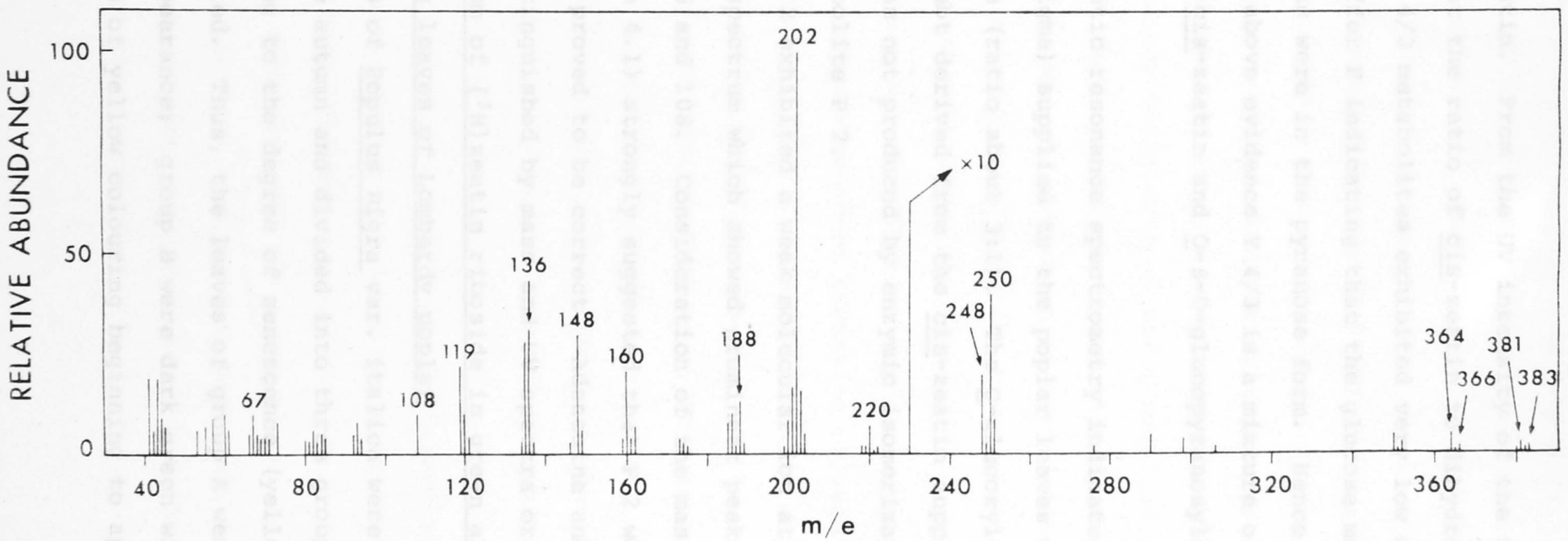
O-β-D-glucopyranosylzeatin was assigned to metabolite Y 3/2.

4.3.3.4. Metabolite Y 4/3.

This metabolite also gave a positive reaction to the enzyme glucose oxidase after hydrolysis. The UV spectral data (Table 4.1) were almost identical to those obtained for Y 3/2 and hence indicated that the metabolite was a 6-(monosubstituted amino)purine. Examination of the mass spectrum (Fig. 4.8.) and comparison of it with the spectrum of Y 3/2 (Fig. 4.7.) suggested that Y 4/3 was a mixture which contained both a zeatin glucoside and a dihydrozeatin glucoside. The mass spectrum showed a pair of weak peaks at m/e 381 and 383 attributable to molecular ions, M-OH peaks at m/e 364 and 366, and M-CH_2OH peaks at 350 and 352. Furthermore, the pair of peaks at m/e 248 and 250 could be assigned to the B+30 fragment ions produced by glucosides of zeatin and dihydrozeatin, respectively. The prominent peaks at m/e 160 and 162 are characteristic of zeatin and dihydrozeatin, respectively.

Hydrolysis of Y 4/3 with β-glucosidase followed by TLC in a two-dimensional system (solvent A, solvent H two times) showed that the hydrolysate contained two UV absorbing components, one exhibited the chromatographic properties of zeatin, the other of dihydrozeatin. In a second two-dimensional TLC system (solvent H three times, solvent G two times) which separates zeatin, cis-zeatin and dihydrozeatin, the presence of dihydrozeatin in the hydrolysate was confirmed and the second UV-absorbing component was found to be chromatographically indistinguishable from

Fig. 4.8. The mass spectrum of the poplar metabolite Y 4/3.


RELATIVE ABUNDANCE
100
50
0
40
80
120
160
200
240
280
320
360
m/e
202
×10
136
148
250
248
188
119
160
67
108
220
364
381
366
383

authentic cis-zeatin. From the UV intensity of the two, it was estimated that the ratio of cis-zeatin to dihydrozeatin was 3:1. Both Y 4/3 metabolites exhibited very low mobility during HVE in buffer F indicating that the glucose moieties of both components were in the pyranose form. Hence, on the basis of the above evidence Y 4/3 is a mixture of O-β-D-glucopyranosyl-cis-zeatin and O-β-D-glucopyranosyldihydrozeatin.

Nuclear magnetic resonance spectrometry indicated the zeatin (source Sigma) supplied to the poplar leaves was a trans-cis mixture (ratio about 3:1). The O-glucosyl-cis-zeatin was no doubt derived from the cis-zeatin supplied to the leaves and was not produced by enzymic isomerization.

4.3.3.5. Metabolite P 2.

Metabolite P 2 exhibited a weak molecular ion at m/e 267 in the mass spectrum which showed prominent peaks at m/e 178, 164, 135 and 108. Consideration of the mass and UV spectra (Table 4.1) strongly suggested that P 2 was adenosine. This proved to be correct. Adenosine and P 2 could not be distinguished by mass and UV spectra or by TLC.

4.3.4. Metabolism of [^{3}H]zeatin riboside in green and senescing leaves of Lombardy poplar

Mature leaves of Populus nigra var. italica were collected in late autumn and divided into three groups (A, B and C) according to the degree of senescence (yellowing) that they exhibited. Thus, the leaves of group A were dark green in appearance; group B were dark green with a few small patches of yellow colouring beginning to appear;

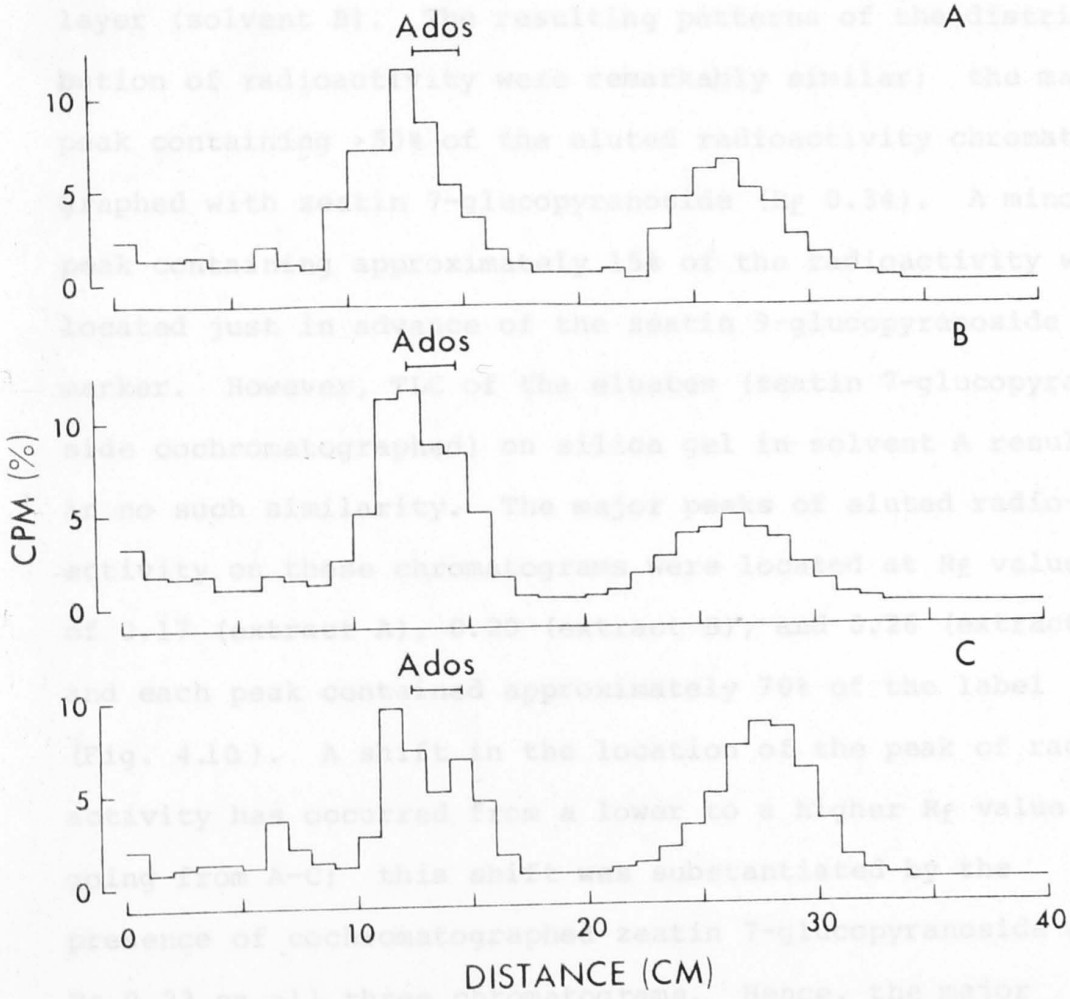
group C were showing definite signs of senescence and were a pale green-yellow colour. Under the conditions described previously, the leaves of each group were supplied with a solution of [8-^{3}H]zeatin riboside (4.0 μM) and extracted after completion of the uptake period (see 4.2.1.). Henceforth, the extracts prepared from the different groups of leaves may be referred to as A, B and C for convenience.

4.3.4.1. Paper chromatography of the crude leaf extract.

Aliquots from each extract were chromatographed on paper in solvent A and each chromatogram was divided into zones for liquid scintillation counting to determine the distribution of radioactivity. The histograms A, B, C of Fig. 4.9. illustrate the distribution for each extract and the location of cochromatographed adenosine on each chromatogram is indicated. Overall, the distribution patterns appear similar, but the small peak located in the R_f region 0.15-0.20 is more prominent in C (yellow) than in A or B. In addition, the R_f region 0.60-0.80 of extract C (the known location of zeatin and zeatin riboside) contains, relatively, a considerably higher level of radioactivity than the same region of A or B.

To enable further investigation of the nature of the metabolites present, preparative paper chromatograms were run for each extract. The major peaks at R_f 0.25-0.40 which encompassed adenosine and the peaks in the R_f region 0.60-0.80 were eluted from each chromatogram with 0.15 M acetic acid. These eluates were evaporated and dissolved in 50% ethanol for chromatography.

Fig. 4.9. The distribution of radioactivity over paper chromatograms (solvent A) of extracts of mature poplar leaves which had been supplied with [8-^{3}H]zeatin riboside. A: extract derived from green leaves, B: extract derived from green leaves beginning to yellow, C: extract derived from yellow, senescing leaves. The barred line Ados denotes the location on each chromatogram of cochromatographed adenosine.


Ados
A
10
5
0
Ados
B
CPM (%)
10
5
0
Ados
C
10
5
0
0
10
20
30
40
DISTANCE (CM)

4.3.4.2. Chromatographic studies of metabolites in the adenosine zone eluates from the paper chromatograms.

Aliquots from all three eluates were cochromatographed with zeatin 7- and 9-glucopyranoside on a silica gel thin layer (solvent B). The resulting patterns of the distribution of radioactivity were remarkably similar; the major peak containing >50% of the eluted radioactivity chromatographed with zeatin 7-glucopyranoside (R_f 0.34). A minor peak containing approximately 15% of the radioactivity was located just in advance of the zeatin 9-glucopyranoside marker. However, TLC of the eluates (zeatin 7-glucopyranoside cochromatographed) on silica gel in solvent A resulted in no such similarity. The major peaks of eluted radioactivity on these chromatograms were located at R_f values of 0.17 (extract A), 0.20 (extract B), and 0.26 (extract C) and each peak contained approximately 70% of the label (Fig. 4.10). A shift in the location of the peak of radioactivity has occurred from a lower to a higher R_f value going from A-C; this shift was substantiated by the presence of cochromatographed zeatin 7-glucopyranoside at R_f 0.23 on all three chromatograms. Hence, the major metabolites present in the paper chromatogram eluates showed a progressive change with advancing senescence. The behaviour of these metabolite complexes in both the paper chromatography and the TLC systems used was similar to that of the zone 3 complex of lupin seedlings (see 3.3.2.) and thus, suggested the possible presence of

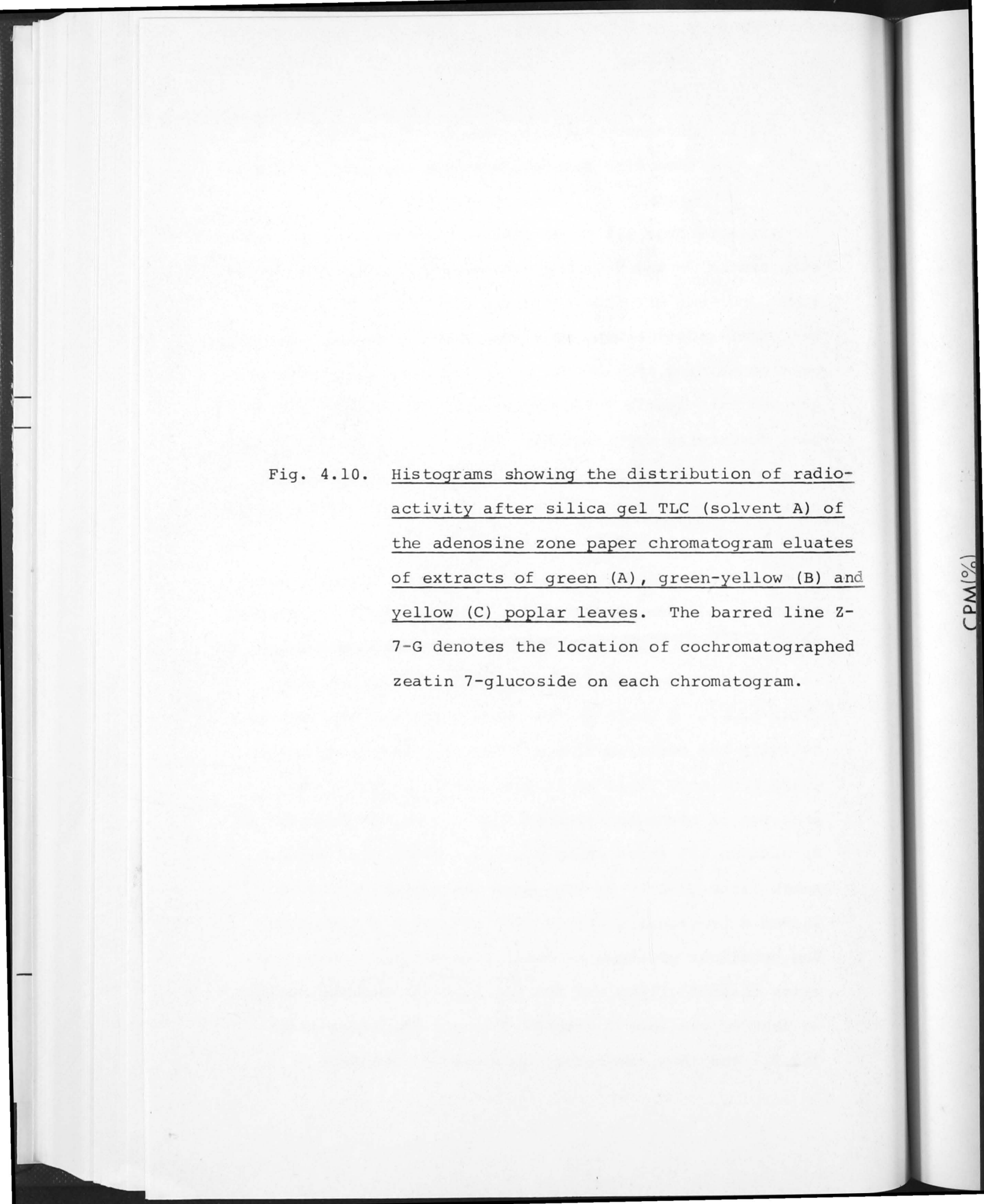
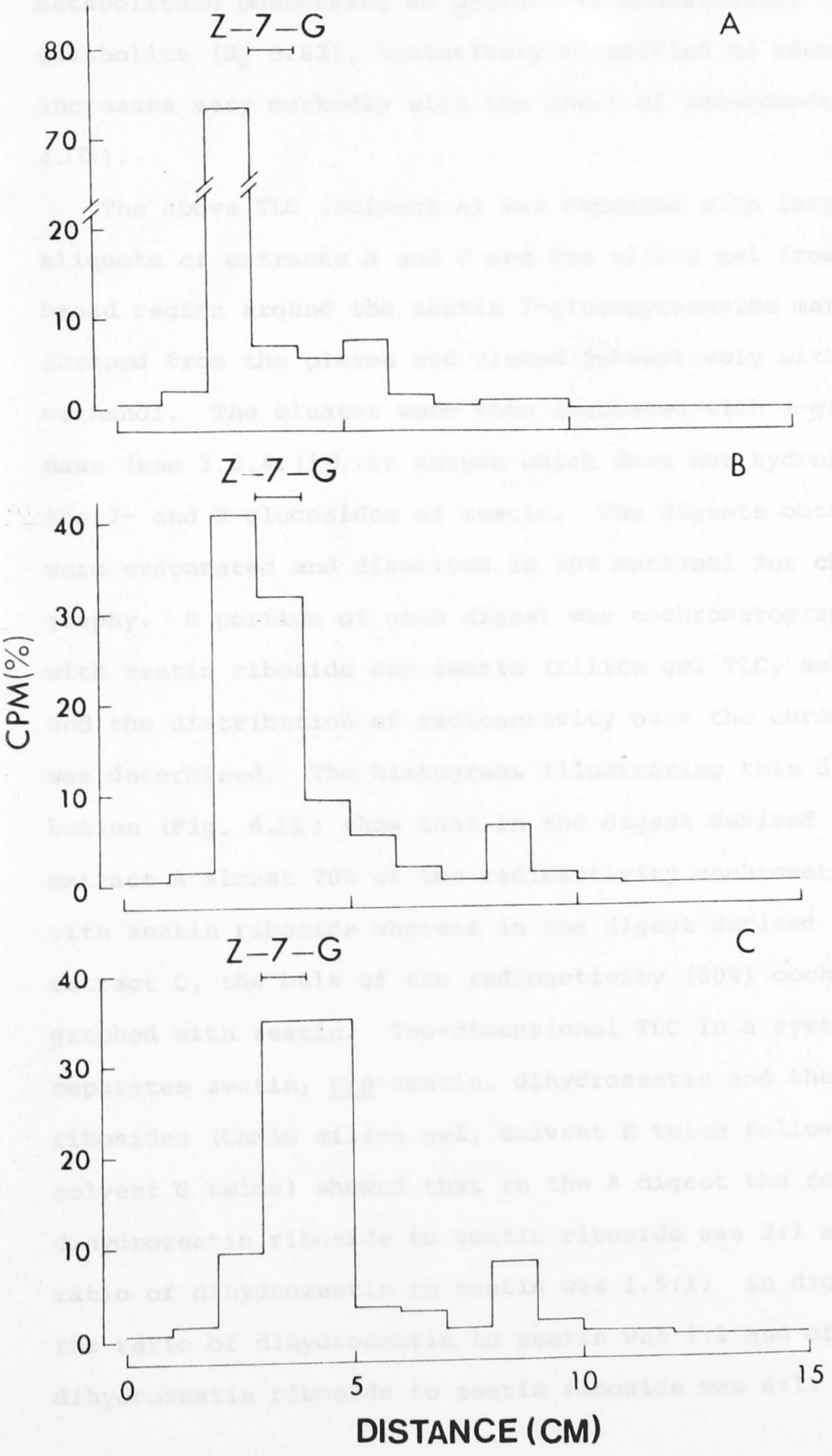


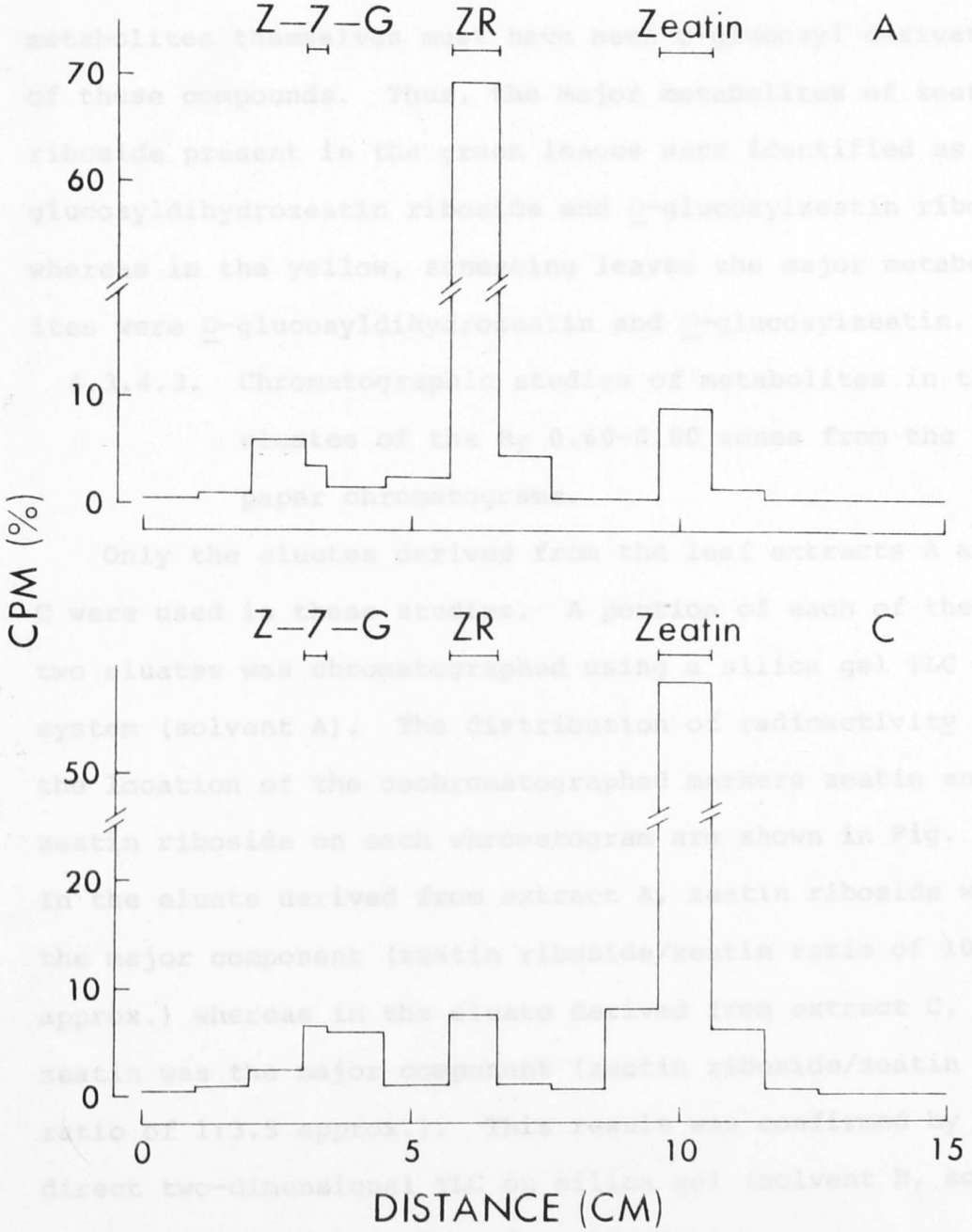
Fig. 4.10. Histograms showing the distribution of radioactivity after silica gel TLC (solvent A) of the adenosine zone paper chromatogram eluates of extracts of green (A), green-yellow (B) and yellow (C) poplar leaves. The barred line Z-7-G denotes the location of cochromatographed zeatin 7-glucoside on each chromatogram.


Z–7–G
A
80
70
20
10
0
Z–7–G
B
40
30
20
10
0
CPM(%)
Z–7–G
C
40
30
20
10
0
0
5
10
15
DISTANCE (CM)

metabolite(s) possessing an O-glucosyl substituent. A minor metabolite (R_f 0.62), tentatively identified as adenine, increases very markedly with the onset of senescence (Fig. 4.10.).

The above TLC (solvent A) was repeated with larger aliquots of extracts A and C and the silica gel from the broad region around the zeatin 7-glucopyranoside marker was scraped from the plates and eluted exhaustively with 80% methanol. The eluates were then incubated with β-glucosidase (see 3.2.4.(i)), an enzyme which does not hydrolyse the 7- and 9-glucosides of zeatin. The digests obtained were evaporated and dissolved in 50% methanol for chromatography. A portion of each digest was cochromatographed with zeatin riboside and zeatin (silica gel TLC, solvent A) and the distribution of radioactivity over the chromatograms was determined. The histograms illustrating this distribution (Fig. 4.11.) show that in the digest derived from extract A almost 70% of the radioactivity cochromatographed with zeatin riboside whereas in the digest derived from extract C, the bulk of the radioactivity (60%) cochromatographed with zeatin. Two-dimensional TLC in a system which separates zeatin, cis-zeatin, dihydrozeatin and their ribosides (CAMAG silica gel, solvent H twice followed by solvent G twice) showed that in the A digest the ratio of dihydrozeatin riboside to zeatin riboside was 2:1 and the ratio of dihydrozeatin to zeatin was 1.5:1; in digest C the ratio of dihydrozeatin to zeatin was 1:1 and of dihydrozeatin riboside to zeatin riboside was 6:1.

4.11A,C. Histograms illustrating the distribution of radioactivity after silica gel TLC (solvent A) of a β-glucosidase digest of the zeatin 7-glucoside region eluates from similar chromatograms to those depicted in Fig. 4.10A, C. A: digest derived from green leaves extract, C: digest derived from yellow leaves extract. The barred lines Z-7-G and ZR denote the location of cochromatographed zeatin 7-glucoside and zeatin riboside, respectively.


Z–7–G
ZR
Zeatin
A
70
60
10
0
CPM (%)
Z–7–G
ZR
Zeatin
C
50
20
10
0
0
5
10
15
DISTANCE (CM)

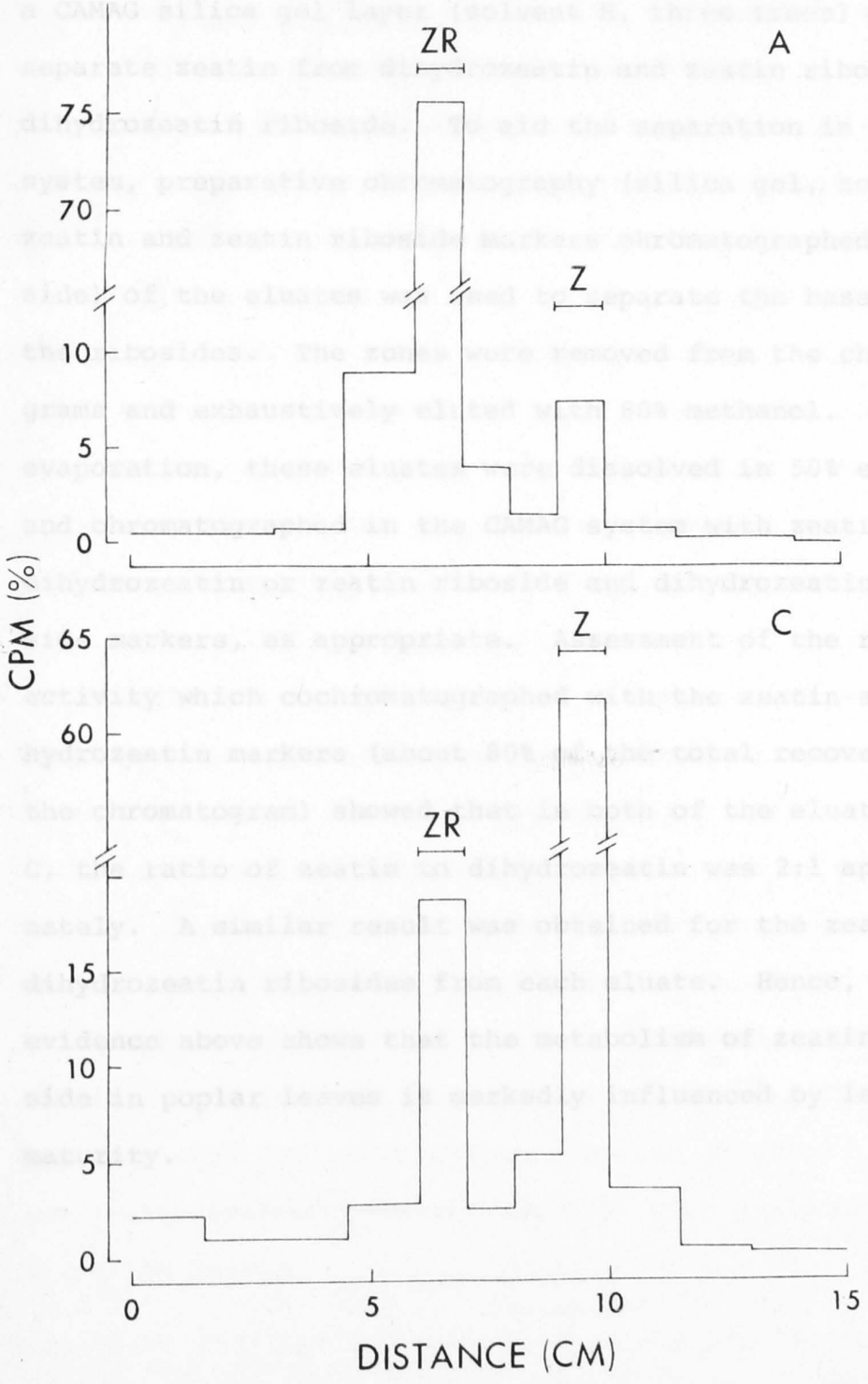
Since the major radioactive metabolites of the adenosine paper chromatogram zones yielded products, on treatment with β-glucosidase, which were chromatographically indistinguishable from the authentic marker compounds zeatin, dihydrozeatin, zeatin riboside and dihydrozeatin riboside, the metabolites themselves must have been O-glucosyl derivatives of these compounds. Thus, the major metabolites of zeatin riboside present in the green leaves were identified as O-glucosyldihydrozeatin riboside and O-glucosylzeatin riboside, whereas in the yellow, senescing leaves the major metabolites were O-glucosyldihydrozeatin and O-glucosylzeatin.

4.3.4.3. Chromatographic studies of metabolites in the eluates of the R_f 0.60-0.80 zones from the paper chromatograms.

Only the eluates derived from the leaf extracts A and C were used in these studies. A portion of each of the two eluates was chromatographed using a silica gel TLC system (solvent A). The distribution of radioactivity and the location of the cochromatographed markers zeatin and zeatin riboside on each chromatogram are shown in Fig. 4.12. In the eluate derived from extract A, zeatin riboside was the major component (zeatin riboside/zeatin ratio of 10:1 approx.) whereas in the eluate derived from extract C, zeatin was the major component (zeatin riboside/zeatin ratio of 1:3.5 approx.). This result was confirmed by direct two-dimensional TLC on silica gel (solvent B, solvent A) of the original crude extracts.

Zeatin and dihydrozeatin and their ribosides are not

Fig. 4.12A,C. Histograms showing the distribution of radioactivity after silica gel TLC (solvent A) of the R_f 0.60-0.80 zone eluates from paper chromatograms of green (A) and yellow (C) poplar leaf extracts. The barred lines ZR and Z indicate the location of cochromatographed zeatin riboside and zeatin markers, respectively.


ZR
A
75
70
Z
10
5
0
CPM (%)
Z
C
65
60
ZR
15
10
5
0
0
5
10
15
DISTANCE (CM)

separated from each other in the chromatography system employed above. However, as described previously, TLC on a CAMAG silica gel layer (solvent H, three times) does separate zeatin from dihydrozeatin and zeatin riboside from dihydrozeatin riboside. To aid the separation in this system, preparative chromatography (silica gel, solvent A; zeatin and zeatin riboside markers chromatographed on one side) of the eluates was used to separate the bases from the ribosides. The zones were removed from the chromatograms and exhaustively eluted with 80% methanol. After evaporation, these eluates were dissolved in 50% ethanol and chromatographed in the CAMAG system with zeatin and dihydrozeatin or zeatin riboside and dihydrozeatin riboside markers, as appropriate. Assessment of the radioactivity which cochromatographed with the zeatin and dihydrozeatin markers (about 80% of the total recovered from the chromatogram) showed that in both of the eluates A and C, the ratio of zeatin to dihydrozeatin was 2:1 approximately. A similar result was obtained for the zeatin and dihydrozeatin ribosides from each eluate. Hence, the evidence above shows that the metabolism of zeatin riboside in poplar leaves is markedly influenced by leaf maturity.

4.4.1. Summary

A study was made of the metabolites formed in mature leaves of Populus alba supplied with [^{3}H]zeatin. Examination of the distribution of radioactivity over a paper chromatogram of the crude extract showed that the major peak of radioactivity was located at R_f 0.28-0.40. Two zeatin metabolites were purified from this zone and were characterized chemically as 6-(4-β-D-glucopyranosyloxy-3-methybutyl-amino)-9-β-D-ribofuranosylpurine (Y 2/1) and O-β-D-glucopyranosylzeatin (Y 3/2); the former compound is a new zeatin metabolite. A third metabolite was found to be a mixture of two compounds which were probably O-β-D-glucopyranosyl-cis-zeatin and O-β-D-glucopyranosyldihydrozeatin (3:1 approximately).

Studies were also undertaken to compare the metabolism of [^{3}H]zeatin riboside in senescing (yellow) and non-senescing (green) leaves of Populus nigra var. italica. A marked difference in metabolism was detected by chromatographic examination and enzymic degradation of extracts prepared from the two types of leaves. Two major, but different, metabolites of zeatin riboside were detected in the different leaves; O-glucosyldihydrozeatin riboside together with O-glucosylzeatin riboside in green leaves, and O-glucosyldihydrozeatin together with O-glucosylzeatin in yellow leaves.

5. DISCUSSION OF RESULTS

In this final chapter, the experimental results of chapters 3, 4 and 5 are discussed collectively.

5.1. TECHNIQUES USED IN CHROMATOGRAPHY AND MASS SPECTROMETRY

A variety of chromatographic methods were employed in this investigation of cytokinin metabolites. Two of these methods (chromatography on cellulose phosphate and TLC using borate-impregnated layers) are not commonly used, while a third (TLC in the presence of a water scavenger) has not been reported previously. These methods are briefly discussed below.

For initial purification of metabolites, column chromatography on cellulose phosphate was employed. To achieve similar purification most cytokinin workers (see e.g. Miller, 1974) employ a polystyrene sulphonic acid resin in the free acid form. Use of cellulose phosphate in the ammonium ion form equilibrated to pH 3.0, as described in the present study has three advantages over sulphonic acid resins in the H^+ form. Firstly, purification is more selective as compounds which are only positively charged at low pH (<3.0) are not retained. All principal cytokinin bases and ribosides (pKa = 3.6-4.0), however, are quantitatively retained on the column and released by dilute ammonia. Secondly, the cytokinins are not exposed to strongly acidic conditions on the cellulose phosphate column. Thirdly, the recovery of cytokinins at the µg level is greater with cellulose based cation exchangers

than with polystyrene derived exchangers.

Although TLC on layers impregnated with borate has proved of limited value in analytical separation of sugar derivatives, the method has not previously been applied to nucleosides and related compounds. In the present study, TLC on borate impregnated silica gel proved invaluable on two occasions. Firstly, as a preparative TLC procedure in the resolution of the cytokinin metabolite complex of lupin seedlings (see 3.2.6.). Secondly, in distinguishing between the 9-glucopyranoside of zeatin and the 9-glucofuranoside (see 2.3.5. and Table 2.2). This separation is based on the ability of the unconstrained 5',6'-diol grouping in the glucofuranosides to form a complex with borate.

In this thesis, the separation of zeatin from dihydrozeatin and zeatin riboside from dihydrozeatin riboside was achieved by TLC on silica gel using a solvent (methylacetate-ethanol, 9:1) to which 2,2-dimethoxypropane and anhydrous formic acid were added to act as a water scavenger (solvent H, see 3.2.3.). The 2,2-dimethoxypropane reacts with water yielding acetone and methanol. Traces of water in the solvent abolish the separation. The separation of zeatin from dihydrozeatin and zeatin riboside from dihydrozeatin riboside has never been achieved previously by TLC. The only previously known method which achieves both of these separations is the new technique of high pressure liquid chromatography (Cole et al., 1974). Gas-liquid chromatography has however been

used to separate zeatin from dihydrozeatin as the TMS derivatives (Most et al., 1968). The TLC method described using solvent H should prove of great value in chromatographic studies of the zeatin group of purine cytokinins. 2,2-Dimethoxypropane, an acetal, has previously been used in organic reactions to remove water. However, its use for this purpose in chromatography has not been reported previously. Traces of water in the solvent and the appreciable amounts held by the silica gel layer would be greatly reduced by reaction with the acetal. The technique may prove of value in separating other classes of compounds.

Mass spectrometry was the key technique for the determination of the structures of the cytokinin metabolites reported in this thesis. The use of mass spectrometry to elucidate the structure of zeatin (Letham et al., 1964) was the first application of this technique to purine structure determination. At that time 0.2 mg of crystalline zeatin was required for one spectrum. As a result of the great advances in instrumentation over the last ten years, mass spectra can now be obtained with 1 μg of sample. Hence, structures could be assigned to the cytokinin metabolites reported in this thesis even though only μg amounts were available. In almost every case the mass spectra presented in this thesis were obtained with underivatized metabolites (e.g. corn metabolite Y, lupin metabolite L 3/C, radish metabolite 19) using the solid sample probe insertion technique, or with TMS derivatives (e.g. lupin metabolite L 2, poplar metabolite Y 2/1) introduced into the mass

spectrometer in the same way. Combined gas chromatography-mass spectrometry, a technique used so successfully with gibberellins, was of negligible value. Thus, mass spectra of TMS derivatives of lupinic acid, O-glucosylzeatin, O-glucosyldihydrozeatin, O-glucosyldihydrozeatin riboside could not be obtained by combined gas chromatography-mass spectrometry (GC-MS) due to thermal decomposition. The TMS derivative of the 3-glucoside of BAP rearranged to the 9-glucoside during passage through the gas chromatograph. Hence, although GC-MS may be useful in identification of cytokinins such as zeatin and zeatin riboside (see e.g. Shindy and Smith, 1975) the technique is not applicable to the more complex purines encountered as metabolites in this thesis.

5.2. IDENTITY OF NEW METABOLITES

The purification of a number of new cytokinin metabolites is reported in this thesis. Those which have been identified are as follows -

corn metabolite Y: zeatin-9-glucopyranoside (probably β sugar linkage)

lupinic acid: β-[6-(4-hydroxy-3-methylbut-trans-2-enylamino)purin-9-yl]alanine

L 3/C: O-β-D-glucopyranosylzeatin

Y 2/1: O-β-D-glucopyranosyl-9-β-D-ribofuranosyldihydrozeatin

i.e. 6-(4-β-D-glucopyranosyloxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine

Y 3/2: O-β-D-glucopyranosylzeatin

Y 4/3 i): O-β-D-glucopyranosyl-cis-zeatin

Y 4/3 ii): O-β-D-glucopyranosyldihydrozeatin

BAP metabolite 19: 6-benzylamino-3-β-D-glucopyranosyl-purine (see Appendix)

A summary of the significant studies of cytokinin metabolism and the identity of the metabolites formed is contained in Table 5.1. This Table is based on the results of this thesis and all literature available at the time of writing. In this section, the identity of the new metabolites detected in the experimental work of this thesis is discussed, with particular reference to their unusual structural features.

Glucoside metabolites of cytokinins which have been identified in addition to those listed above include the 7-glucoside of zeatin (Parker and Letham, 1972, 1973) and the 7- and 9-glucosides of BAP (Parker et al., 1973; Wilson et al., 1974; Fox et al., 1973). The 3- and 7-glucoside metabolites of cytokinins are unusual in two respects - the site of the glycosidic linkage and the sugar involved. The only previously known purine 7-glycosides were certain compounds related to vitamin B_{12} which contain a 7-ribosylpurine moiety. The 3-glucoside of 6-benzylaminopurine is the first compound with a glycosidic linkage at position 3 of a purine ring to be isolated from a plant tissue. Only two compounds of this type have previously been purified from natural sources; these are 3-ribofuranosyluric acid and its 5'-phosphate, both obtained from beef blood (Forrest et al., 1961; Hatfield

TABLE 5.1

A list of the principal studies of cytokinin metabolism in plants and the metabolites identified

Cytokinin, plant tissue and reference	Metabolites identified
6-BAP[1], *Xanthium pensylvanicum* leaf disks, (McCalla *et al.*, 1962)	N_6-benzyladenosine[2], N_6-benzyladenylic acid, adenylic, guanylic and inosinic acids, adenine, guanine, urea and a ureide
6-BAP, *Cicer arietinum* seedlings (Guern *et al.*, 1968)	N_6-benzyladenosine[2]
6-BAP, *Lemna minor* (Bezemer-Sybrandy and Veldstra, 1971)	N_6-benzyladenosine 5'-phosphates
6-BAP, *Solanum andigena* stolons and stems (Woolley and Wareing, 1972a,b)	N_6-benzyladenosine
6-BAP, soybean callus, potato tuber, tobacco tissue cultures (Dyson *et al.*, 1972; Fox *et al.*, 1973)	7-glucosyl-6-BAP[2], N_6-benzyladenosine, N_6-benzyladenosine 5'-monophosphate
6-BAP, radish cotyledons (Wilson *et al.*, 1974; Letham *et al.*, 1975)	7-glucosyl-6-BAP[2], 9-glucosyl-6-BAP, N_6-benzyladenosine, 3-glucosyl-6-BAP
6-BAP and kinetin, *Acer pseudoplatanus* cell suspensions (Doree and Guern, 1973; Guern *et al.*, 1974)	N_6-benzyladenosine 5'monophosphate[2] and kinetin riboside 5'-monophosphate[2], ureides, nucleotides of adenine
Z[3], bean axes (Sondheimer and Tzou, 1971)	(diH)Z, (diH)[9R]Z, (diH)[9R 5'P]Z, [9R]Z, [9R 5'P]Z
Z, ash embryos (Tzou *et al.*, 1973)	[9R]Z[2], [9R 5'P]Z, 5'-di- and tri-phosphates of [9R]Z
Z, de-rooted radish seedlings (Parker *et al.*, 1972; Parker and Letham, 1973)	7-glucosylzeatin[2], [9R 5'P]Z, [9R]Z, adenine, adenosine and AMP
Z, radish roots (Gordon *et al.*, 1974)	7-glucosylzeatin, [9R 5'P]Z, [9R]Z

Cytokinin, plant tissue and reference	Metabolites identified
Z, *Zea mays* seedlings (Parker and Letham, 1974; Parker *et al.*, 1973; this thesis)	9-glucosylzeatin, [9R 5'P]Z, [9R]Z, adenine, adenosine, adenosine 5'-phosphate, and 7-glucosylzeatin (minor metabolite)
Z, de-rooted lupin seedlings (Parker *et al.*, 1975; MacLeod *et al.*, 1975; this thesis)	*O*-β-D-glucopyranosylzeatin, β-[6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purin-9-yl]-alanine, (diH)Z
Z, *Populus alba* leaves (this thesis)	*O*-β-D-glucopyranosylzeatin, *O*-β-D-glucopyranosyldihydrozeatin, 9-β-D-ribofuranosyl-*O*-β-D-glucopyranosyldihydrozeatin, (diH)Z
iP, tobacco and *Acer* cell suspensions, (Laloue *et al.*, 1974)	5'-mono-, 5'-di- and 5'-tri-phosphates of [9R]iP

[1] 6-benzylaminopurine [2] indicates compound is the principal metabolite [3] zeatin

Other abbreviations: (diH)Z - dihydrozeatin; (diH)[9R]Z - dihydrozeatin riboside; (diH)[9R 5'P]Z - dihydrozeatin riboside 5'-phosphate; [9R]Z - zeatin riboside; [9R 5'P]Z - zeatin riboside 5'-phosphate; iP - isopentenyladenine; [9R]iP - isopentenyladenosine

and Forrest, 1962). The identification of glucosides of zeatin and BAP was the first unequivocal evidence for the occurrence of purine glucosides in living tissues.

The 9-glucoside of zeatin is the first purine derivative isolated from a higher plant with a sugar other than ribose at position 9. However, micro-organisms produce purine derivatives with unusual sugars at position 9. These sugars include 3'-deoxyribose (as in cordycepin), ketohexoses (as in angustmycin A and C), arabinose (as in spongoadenosine), and 4-fluoro-5-O-sulfamoylpentofuranose (as in nucleocidin) (see Suhadolnik, 1970). However, 9-glucosyl purines do not appear to have been isolated from micro-organisms. From sweet corn, a 9-glycoside of zeatin, which differed from zeatin riboside, was purified (Letham, 1973b). The sugar was not identified. However, its chromatographic properties resemble those of zeatin-9-glucoside identified as a metabolite of zeatin (see 2.3.) in Zea mays roots and it is possible that the two compounds are identical.

This thesis reports the first identification of O-glucosyl metabolites of zeatin. Five compounds of this type have been listed above. These metabolites, unlike the 3-, 7- and 9-glucosides, are not structurally unique. A great diversity of O-glucosides occur in nature. However O-glucosyldihydrozeatin riboside (Y 2/1) is the most complex zeatin metabolite identified to date.

Although the N 7- and 9-glucosides of zeatin were identified as metabolites in all the species studied in this thesis, and the 7-glucoside is a known metabolite in radish, the O-glucosyl metabolites did not appear to occur so widely. They were detected only in lupin seedlings and poplar leaves; they were not detected in corn seedlings and also probably do not occur in radish seedlings. Thus, there would appear to be some species specificity with regard to their occurrence.

It is interesting to note that in extracts of Populus x robusta leaves, Hewett and Wareing (1973c) have detected a cytokinin which, like O-glucosylzeatin, is readily hydrolysed by β-glucosidase and exhibits chromatographic behaviour on Sephadex LH20 identical to that of O-glucosylzeatin. Thus, this naturally occurring cytokinin and O-glucosylzeatin are probably identical.

The structure of the sugar moieties of the cytokinin glucoside metabolites have been investigated. In the case of the 7- and 9-glucosides, including zeatin 9-glucoside, the metabolites have been compared with glucopyranosides and glucofuranosides prepared by unambiguous synthesis (Cowley et al., 1975; Duke et al., 1975). These comparisons have shown that the glucose moieties of both the 7- and 9-metabolites are in the pyranose form. Borate buffer HVE (pH 8.25) was used to determine the ring structure of the glucose moiety in the O-glucosylzeatin metabolites (L 3/C, Y 3/2, Y 4/3). Subsequent direct comparison with the synthetic compounds confirmed the pyranose structure assigned; the synthetic compounds and the metabolites were indistin-

guishable in this system. It should be noted that Deleuze et al. (1972) proposed a furanose structure for the 7-glucoside metabolite of 6-benzylaminopurine isolated from potato tuber tissue. However, this assignment is based entirely on a comparison of fragment ion intensities in the mass spectrum of the metabolite (TMS derivative) with those in the spectra of simple glucopyranosides and glucofuranosides. Hence, it lacks an acceptable experimental basis. In further work in this laboratory, the metabolism of BAP was studied in potato tuber tissue. The principal metabolite, without doubt the compound purified by Fox and co-workers (1973), was shown to be the 7-glucopyranoside. In addition, the 9-glucopyranoside was also identified as a metabolite. It is also of interest that in studies in this laboratory it was found that radish seedlings did not form 7- and 9-glucosides from exogenously supplied labelled adenine (Letham, unpublished results). Hence 7- and 9-glucosylation may be restricted to cytokinins and perhaps other N_6-substituted adenines.

The configuration of the glycosidic linkage in zeatin 9-glucoside was not established. However, the 9-α- and 9-β-glucopyranosides of BAP were synthesised by collaborators in the Research School of Chemistry and, by chromatographic methods, the 9-glucoside metabolite of BAP was found to be identical to the synthetic β-glucoside and to differ from the α-glucoside. Hence, by analogy, the 9-glucoside of zeatin probably possesses a β-linkage. It should be noted that 7- and 9-glucosides of purines are

not cleaved by glucosidase and therefore it is not possible to use enzymic methods to determine configuration of glucosidic linkages in these compounds.

The purinyl-amino acid conjugate, termed lupinic acid, which was identified as a metabolite of zeatin in Lupinus angustifolius seedlings is also structurally unusual. It is the first reported, naturally occurring, purine derivative in which an amino acid moiety is conjugated to a purine ring nitrogen atom. However, pyrimidinyl amino acids (willardiine and isowillardiine) are known (Ashworth et al., 1972). There are reports in the literature concerning the purification from plants of enzyme activity capable of catalysing the addition of an amino acid moiety (alanine) to N atoms of other heterocyclic ring systems (see 5.3.). At the present time, lupinic acid has been identified only as a zeatin metabolite in lupin seedlings and has been shown not to be a metabolite in poplar leaves. However, minor peaks of unidentified metabolites which are located at appropriate R_f values on paper chromatograms of corn and radish extracts may contain lupinic acid. Recently, in this laboratory, the 9-alanine conjugate of 6-benzylaminopurine has been identified as a metabolite formed from BAP in bean seedlings (Letham, unpublished data); this is probably the unidentified bean metabolite referred to by McCalla et al. (1962) in the first reported study of cytokinin metabolism.

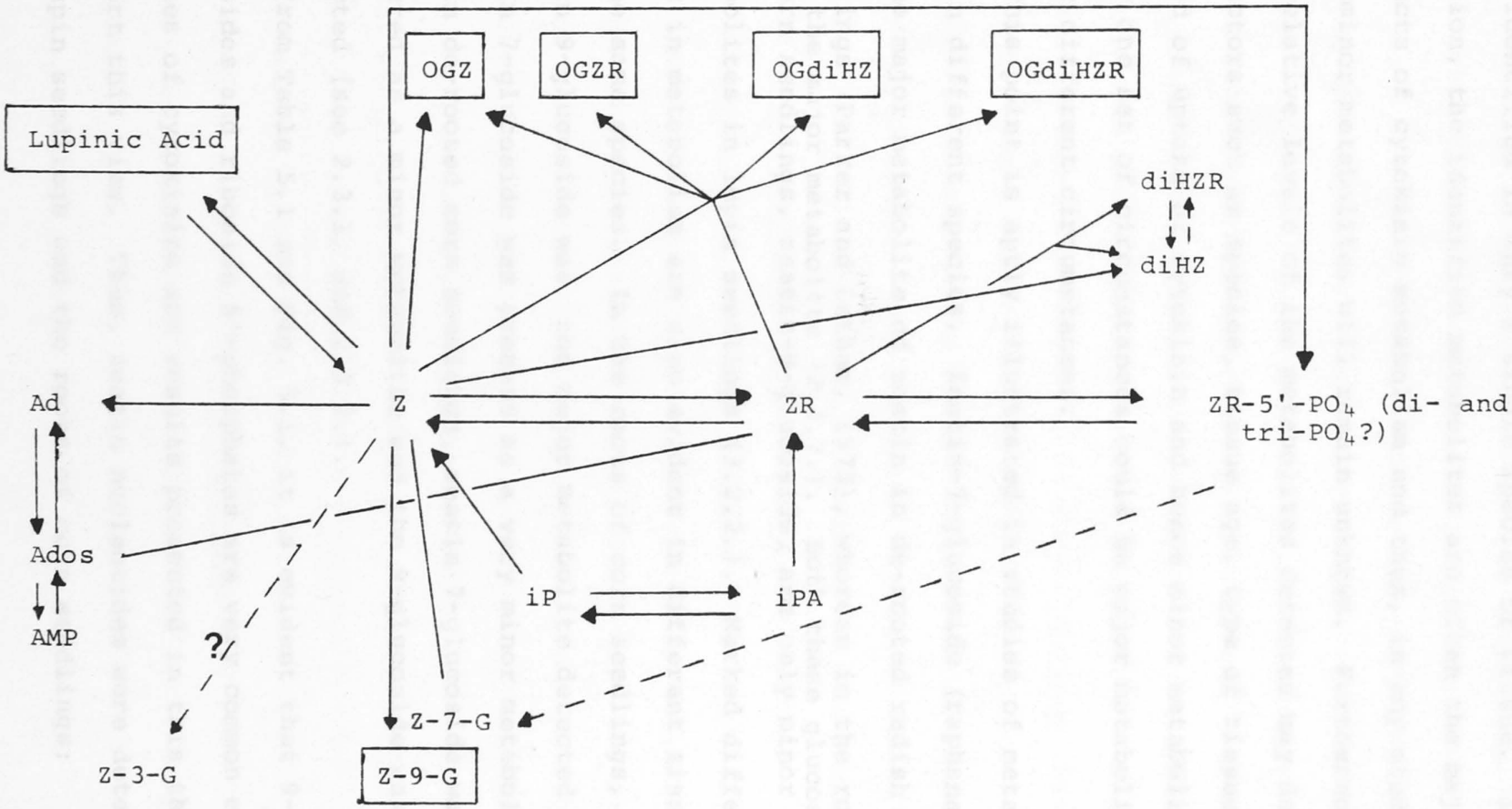
The isoprenoid unit occurs widely as a basic building block in biosynthesis of a diversity of compounds and the

double bond contained in it is often subsequently saturated. Up until the time of writing, excised bean axes (Phaseolus vulgaris) were the only tissue reported to metabolize exogenously supplied zeatin to dihydrozeatin and its riboside and ribotide derivatives (Sondheimer and Tzou, 1971). However, results reported herein (see 3.3. and 4.3.) show that lupin and poplar species are also able to reduce the zeatin side chain; in the case of poplar extensive metabolism of the dihydrozeatin itself appears to occur, e.g. formation of O-glucosyldihydrozeatin riboside. Dihydrozeatin has been identified as an endogenous cytokinin in lupin seeds (Koshimizu et al., 1967). Radish (Parker and Letham, 1973) and ash (Tzou et al., 1973) did not metabolize zeatin to dihydrozeatin. Thus, the saturation of the isopentenyl side chain of cytokinins is another aspect of metabolism which exhibits species specificity.

5.3. PATHWAYS OF CYTOKININ METABOLISM

The scheme in Fig. 5.1. represents a summary of the present knowledge regarding the metabolism of exogenously supplied zeatin and zeatin riboside in a variety of plant tissues. The scheme is not a metabolic pathway in the true sense as, in most cases, the intermediates which are probably formed between the supplied zeatin and the identified metabolites have not been characterised. However, in certain instances (e.g. metabolism of zeatin to O-glucosyldihydrozeatin riboside) the identity of likely intermediates can be suggested. Also, it should be pointed out that the scheme represents a compilation of data from

Fig. 5.1. Scheme summarising present knowledge of the metabolism of naturally occurring cytokinins in various plant tissues. Broken line indicates a reaction which probably occurs. Question mark indicates that the 3-glucoside of zeatin has not yet been identified. Abbreviations used: Z - zeatin; ZR - zeatin riboside; ZR-5'-PO_4 - zeatin riboside 5'-monophosphate; Z-3, 7 or 9-G - zeatin 3, 7 or 9-β-D-glucopyranoside; diHZ - dihydrozeatin; diHZR - dihydrozeatin riboside; OGZ - O-β-D-glucopyranosylzeatin; OGZR - 9-β-D-ribofuranosyl-O-β-D-glucopyranosylzeatin; OGdiHZ - O-β-D-glucopyranosyldihydrozeatin; OGdiHZR - 9-β-D-ribofuranosyl-O-β-D-glucopyranosyldihydrozeatin; Ad - adenine; Ados - adenosine; AMP - adenosine 5'-monophosphate; iP - isopentenyladenine; iPA - isopentenyladenosine.



*New metabolites identified during the course of the work for this thesis are indicated by 'boxing in'.

many different studies and some of the metabolites have been identified in only a single species or tissue. In addition, the identified metabolites are often the major products of cytokinin metabolism and thus, in any study, many minor metabolites will remain unknown. Furthermore, the relative levels of the metabolites detected may depend on factors such as species, tissue age, type of tissue and period of uptake of cytokinin and hence minor metabolites under one set of circumstances could be major metabolites under different circumstances.

This point is aptly illustrated in studies of metabolism in different species. Zeatin-7-glucoside (raphanatin) is the major metabolite of zeatin in de-rooted radish seedlings (Parker and Letham, 1973), whereas in the roots of corn seedlings, zeatin-9-glucoside is the major metabolite (2.3.7.). Both these glucosides are only minor metabolites in lupin seedlings (3.2.2.). Marked differences in metabolism are also evident in different tissues of the same species. In the roots of corn seedlings, zeatin 9-glucoside was the major metabolite detected and zeatin 7-glucoside was present as a very minor metabolite, but in de-rooted corn seedlings, zeatin 7-glucoside was detected as a minor metabolite and the 9-glucoside was not detected (see 2.3.1. and 2.3.2.).

From Table 5.1 and Fig. 5.1. it is evident that 9-ribosides and riboside 5'-phosphates are very common metabolites of cytokinins and results presented in this thesis support this view. Thus, zeatin nucleotides were detected in lupin seedlings and the roots of corn seedlings;

adenine nucleotides were present in roots of corn seedlings, lupin seedlings and mature poplar leaves; zeatin riboside was detected in all three tissues. In Fig. 5.1. the question of whether the 5'-phosphates are formed by a single-step reaction or by a two-step reaction (i.e. ribosylation followed by phosphorylation) is also raised. It is possible to prepare enzymically nucleoside 5'-monophosphates from N_6-substituted adenosines and ATP, the kinase activity required being present in both yeasts and higher plants (Doree and Terrine, 1973). These *in vitro* enzyme reactions suggest that cytokinin nucleotide biosynthesis from the free bases may be a two-step process involving first a nucleoside phosphorylase to yield a cytokinin riboside and then a kinase to give a nucleotide. However in cells of *Acer pseudoplatanus* at least, this does not appear to occur. In these cells the well established *in vivo* formation of cytokinin riboside 5'-monophosphates from the corresponding bases appears to involve the direct transfer of a riboside 5'-monophosphate group from 5-phosphoribosylpyrophosphate to the free cytokinin base catalysed by a purine phosphoribosyltransferase. Cell extracts of *Acer pseudoplatanus* catalyse such a reaction from adenine, 6-methylaminopurine and 6-benzylaminopurine (Pethe-Sadorge *et al.*, 1972; Doree and Guern, 1973). Thus, by analogy, a similar process could also result in the one-step formation of zeatin riboside 5'-monophosphate from zeatin.

The conversion of cytokinins and their ribosides to adenine and adenosine is also a common metabolic fate (see Fig. 5.1., Table 5.1). All of the species studied in this thesis were found to contain adenine and adenosine derived from the exogenously supplied cytokinin, but again their relative importance as metabolites appear to be linked to species and tissue type. Thus, they are minor metabolites of zeatin in poplar leaves, de-rooted seedlings of lupin and also radish (Parker and Letham, 1973) but major metabolites in de-rooted corn seedlings. However, they are only minor metabolites of BAP when supplied to de-rooted corn seedlings (see below and Table 2.3). Enzymic activity capable of catalysing such reactions (isopentenyladenosine as substrate) has been found in crude preparations from tobacco tissue (Paces et al., 1971). More recently, a similar enzyme has been purified from corn kernels which is specific for the naturally occurring cytokinins zeatin riboside and isopentenyladenosine or their free bases (Whitty and Hall, 1974). This enzyme was found to require oxygen and hence was categorised as an oxidase. The oxidase preparation was isolated in company with a nucleoside hydrolyase. Detailed examination of the oxidase reaction revealed that isopentenyladenine was degraded to yield an aldehydic derivative of the side chain together with adenine (Brownlee et al., 1974).

As suggested in Fig. 5.1. there is some evidence which indicates that zeatin riboside 5'-monophosphate may be the immediate precursor of zeatin 7-glucoside. Gordon et al.

(1974), in a study of zeatin metabolites in radish roots, provided evidence that soon after commencement of uptake the major metabolite detected was zeatin riboside 5'-monophosphate and that, with time, the level of this metabolite declined as that of zeatin 7-glucoside increased. Similar evidence has been presented by Fox *et al.* (1973) based on studies of BAP metabolism involving several different tissues. This evidence is consistent with, but does not conclusively establish, the existence of a precursor-product relationship between nucleotide and 7-glucoside.

In many metabolism studies (see Table 5.1), the exogenously supplied hormone is the synthetic cytokinin, 6-benzylaminopurine (BAP). In some species, the identity and relative levels of metabolites formed from BAP and zeatin are analogous. Thus, when supplied to radish seedlings both cytokinins are rapidly metabolized, possibly *via* nucleotides, to the 7-glucosides as the major stable products (Parker and Letham, 1973; Wilson *et al.*, 1974; Gordon *et al.*, 1974). However, in contrast, the metabolism of these two cytokinins in *Zea mays* seedlings is markedly different. Zeatin is readily metabolized, being converted to zeatin 7-glucoside (9-glucoside a minor metabolite), nucleotides, adenine and adenosine in about equal amounts. On the other hand, BAP appeared to be much more stable than zeatin (approximately 20% extracted radioactivity identified as BAP, cf. <2% for zeatin) and was metabolized to a single major product, BAP-9-glucoside (2.3.6. and Table 2.3). Also, adenine and adenosine were quite minor

metabolites of BAP (cf. zeatin) and this might be explained in terms of BAP being an unsuitable substrate for the oxidase/hydrolyase complex discussed above. Overall, these results suggest that in the time course of the experiment (70 hours) the capability to metabolize BAP in the amounts supplied is lacking or non-functional in the corn seedlings.

In the introduction to chapter 2, it was suggested that changes in the endogenous cytokinin level might constitute the principal hormonal regulatory mechanism in the leaves of the Gramineae. The results in this thesis indicate that metabolism to the 7- and 9-glucosides, adenine and adenosine is one mechanism by which endogenous cytokinin level may be regulated.

The zeatin-amino acid conjugate, lupinic acid, is unique among the conjugated metabolites because instead of being joined to a glycosidic group, zeatin is linked with an alanine moiety (see Fig. 5.1.). As will be discussed below, conjugation with an amino acid moiety is known to occur with auxins, but this is the first such cytokinin (purine) metabolite to be reported. There are several reports in the literature of enzyme activity capable of catalysing (*in vitro*) the addition of an alanine moiety to an N atom of a heterocyclic nucleus other than purine (see e.g. Murakoshi *et al.*, 1975; Murakoshi *et al.*, 1973; Ashworth *et al.*, 1972). This activity has been purified from sources such as watermelon, pea and sweet pea and in all cases the alanine substituent was derived from *O*-acetyl-L-serine; neither serine nor *O*-phosphoserine could

serve as donors of the alanine moiety. Using a crude acetone powder preparation from lupin seedlings, the author attempted the *in vitro* synthesis of lupinic acid from [G-^{3}H]zeatin and *O*-acetyl-L-serine. The data obtained suggested that the formation of lupinic acid had occurred, but in extremely small amounts. Hence the alanine moiety of lupinic acid is probably derived from *O*-acetyl-serine.

The *in vitro* biosynthesis of ribosides and nucleotides and its possible relationship to *in vivo* metabolism of cytokinins has been discussed above. Another common metabolic fate of cytokinins is conjugation with glucose, such compounds being identified in all of the species studied in this thesis. In *Zea mays* roots, the 9-glucoside was the principal metabolite of zeatin. In addition, glucosylation of the synthetic cytokinin, 6-benzylaminopurine, has also been reported to occur widely (Fox *et al.*, 1973). Little is known of the enzymes which catalyse these reactions or the glucose donors involved. The most likely candidate for the role of glucose donor is uridine diphosphate glucose (UDPG). In fact, recent studies in this laboratory (Letham and Wilson, unpublished data) have shown that UDPG can function as a glucose donor in the biosynthesis of BAP-7-glucoside from BAP, using a crude cell-free enzyme preparation from radish. Less conclusive evidence suggested that BAP-9-glucoside was also formed in much smaller amounts. Thus, it is possible that the zeatin-9-glucoside formed in roots of corn seedlings might also be synthesised *in vivo* using UDPG as a glucose donor.

UDPG has already been reported to act as a glucose donor for the *in vitro* synthesis of the glucosides of the heterocyclic compound, isoxazolinone, and its derivatives. The enzymes which catalysed these syntheses were contained in preparations from pea, sweet pea and watermelon (Murakoski *et al.*, 1975; Murakoski *et al.*, 1974).

The metabolites of cytokinins formed by animal tissues and the enzymes involved have also been studied. No doubt, a substantial impetus to this work was the discovery that cytokinin ribosides inhibit growth of animal tumour cells and induce remissions in human leukaemia patients. The metabolism of intravenously administered isopentenyladenosine (radioactively labelled) in humans has been studied; identified metabolites included 6-*N*-(3-methyl-3-hydroxybutylamino)purine, hypoxanthine and adenine. A large proportion of the radioactivity was excreted as non-UV-absorbing compounds suggesting degradation of the purine moiety (Chheda and Mittelman, 1972). However, at the time of writing, there had been no reports of the identification in animal tissues of amino acid- or glucose-cytokinin conjugates such as are now known to be formed in plant tissues.

Metabolites formed by the conjugation of the supplied hormone with glucose or an amino acid moiety are not unique to cytokinins. The metabolism of auxins, gibberellins and abscisic acid has been studied in various plant tissues; all three types of hormones form glucosides, and, in addition, auxins form amino acid conjugates. The

auxin indole-3-acetic acid (IAA) has been shown to form a conjugate with aspartic acid, indole-3-acetylaspartic acid (IAA-Asp), in pea seedlings (Andreae and Good, 1955). The synthetic auxin, naphthalene acetic acid, has also been shown to form such a conjugate (Schraudolf, 1971). Also, a conjugate of IAA with lysine, indole-3-acetyl-L-lysine, has been identified in the culture medium of the bacterium, Pseudomonas savastanoi (Hutzinger and Kosuge, 1968). While these auxin metabolites are similar to the zeatin metabolite, lupinic acid, in that they all involve conjugation with an amino acid moiety, the chemical nature of the linkage is quite different. In lupinic acid, the β-carbon of the alanine moiety is joined to the purine ring at N9, whereas in the auxin metabolites, amino-acid amino groups are joined to the carbonyl group of the auxin in an amide linkage. The IAA-glucose conjugate, indole-3-acetyl-β-D-glucosyl-ester, identified independently as an auxin metabolite by Klambt (1961) and Zenk (1961) also differs chemically from its cytokinin counterparts in the nature of the linkage. The auxin metabolite is a glucosyl-ester but the cytokinin-glucose conjugates are the result of a β-linkage of the glucose to the nitrogen atom at the 3, 7 or 9 positions of the purine ring or to the hydroxyl of the zeatin side chain. Similarly, the abscisic acid (ABA)-glucose conjugate, first identified in tomato fruits (Milborrow, 1967), is also the result of a glucosyl-ester linkage to the ABA moiety.

As yet, unlike auxins and cytokinins, no amino acid

conjugates of gibberellins have been reported. However the gibberellins are known to be metabolized to form conjugates with glucose. Thus GA_8-*O*(2)-β-D-glucopyranoside has been purified and identified from maturing fruits of *Phaseolus coccineus* (Schreiber *et al.*, 1967, 1970), seeds of *Pharbitis nil* (Tamura *et al.*, 1968) and apices of *Althaea rosea* (Harada and Yokota, 1970). Many such conjugates of the different gibberellins have now been reported. As in the case of the *O*-glucosyl zeatin metabolites from lupin and poplar (this thesis: 3.3. and 4.3.), in some gibberellin metabolites the glucose moiety is linked to the oxygen of a hydroxyl group. However, as with IAA, the gibberellins are also metabolized to glucosyl-esters, the ester linkage being at the 7-COOH of the GA molecule.

Thus, glucose conjugates are formed as metabolites from all four types of plant hormones when supplied exogenously to various plant species. However, glucosides of auxins, abscisic acid and gibberellins are all *O*-glucosyl compounds in which the glucose engages a hydroxyl or carboxyl group of the hormone; this type of glucoside occurs commonly in plants. In contrast, cytokinins form purine *N*-glucosyl metabolites; these are structurally unique among natural products related to nucleic acids. They are the only known natural compounds in which a hexose sugar is linked to a purine or pyrimidine ring nitrogen. Although glucose is known to occur conjugated to other heterocyclic nitrogen atoms (e.g. the 2 position of 3-isoxazolin-5-one), such compounds are extremely rare.

5.4. CYTOKININ METABOLITES: PROPERTIES AND SIGNIFICANCE IN RELATION TO SOME PHYSIOLOGICAL PROBLEMS

The studies of cytokinin metabolism reported in this thesis are not to be regarded as an end in themselves. Rather, it is hoped they will help in providing a chemical basis on which certain physiological studies relating to plant growth and development may be based. Studies of metabolism could, of course, lead in a more direct way to an understanding of important aspects of cytokinin biochemistry and function. The above matters are briefly discussed in this section and the relevance of the results of this thesis to the solution of physiological problems involving cytokinins is assessed.

The importance of metabolism studies in relation to storage forms of cytokinins has already been mentioned (see 1.7.). In many instances, the "bound" forms often referred to in the literature might actually be storage forms of cytokinins. These storage forms (probably inactive *per se*) may provide a physiological reserve of inactive (but readily activated) cytokinin which would be available to participate in regulation of developmental processes when required during different phases of the plant's life cycle. Purified aqueous extracts (inhibitors removed) prepared from the mesocarp of avocado fruits were inactive in a cytokinin bioassay but activity appeared after acid hydrolysis (Gazit and Blumenfeld, 1970). In addition, the level of this bound cytokinin activity was found to vary with the stage of fruit development, being high in

young rapidly growing fruit and diminishing with the reduction in rate of fruit growth. Other plant organs in which bound cytokinins have been detected are the flower and the seed. Quite different levels of extractable cytokinins were detected in pistillate and staminate flowers of cucumber; prior to acid hydrolysis, only the ethanolic extracts of pistillate flowers contained cytokinin activity, but following acid hydrolysis, the staminate flower extract also showed cytokinin activity (Borkowska and Borkowski, 1975). Variations in the endogenous levels of free and bound cytokinin activity have also been observed in apple seed during the period of stratification (Borkowska and Rudnicki, 1975). Most of the cytokinin activity of dry, non-stratified seed was present in the bound form, being detectable only after acid hydrolysis of the extract. After commencement of stratification, the level of this bound activity decreased initially, then returned almost to the initial level after 5 weeks and then finally again declined steadily until the completion of stratification. On the other hand, the level of free cytokinin, initially very low, showed a dramatic increase in the first 5 weeks of stratification after which the level also gradually declined (Borkowska and Rudnicki, 1975). Although the above reports demonstrate the existence of bound cytokinins, no attempt at identification of the bound forms was made. However, Yoshida and Oritani (1972) have reported a bound cytokinin in rice plant roots and provided some evidence that it may be a zeatin

glucoside.

Evidence for the existence of bound or storage forms of plant hormones is not confined to the cytokinins (see 5.3.). Gibberellin-glucose conjugates have been identified as metabolites of exogenously supplied gibberellins and have also been shown to occur naturally (see reviews by Lang, 1970 and Sembdner, 1975). The precise physiological role of bound gibberellins is still unclear, but there is evidence for two functions, namely, as storage and transport forms. Evidence for their function as a storage form is mainly suggested by the interconversions between the free and bound forms during seed development and germination (Barendse et al., 1968).

Abscisic acid, now generally regarded as a plant hormone, is also known to occur naturally in a bound form, namely as a glucosyl-ester (see 5.3.). This glucosyl-ester is also the major metabolite of exogenously supplied ABA in various species (e.g. Milborrow, 1970). The glucosyl-ester appears to be of importance in the regulation of growth. Thus a recent report details seasonal changes in the levels of free and bound (probably the glucosyl-ester) abscisic acid in buds of blackcurrant and beech; the highest free/bound ratio occurred in the autumn and the highest bound to free ratio coincided with bud burst (Wright, 1975). In a rather more precise study concerning bud dormancy break in almond, Leshem et al. (1974) showed that successive stages of bud break manifest a marked increase of bound ABA, paralleled by a

decrease in endogenous levels of the free form. Each of the examples cited above serves to confirm that storage or bound forms of cytokinins, gibberellins and abscisic acid do have important physiological roles to play in conjunction with the free forms in plant growth and development.

All of the new cytokinin metabolites possessing an N_6-substituted adenine moiety identified in the studies reported herein are potentially storage forms, with the possible exception of the 3-glucoside of BAP (see appendix I). Zeatin 9-glucoside, the major root metabolite of zeatin in Zea mays seedlings, was still a major metabolite in root tissue 7 days after the supply of labelled zeatin had ceased. Similar persistence or stability had previously been observed for zeatin 7-glucoside (raphanatin), the major zeatin metabolite formed in cotyledons of de-rooted radish seedlings (Parker and Letham, 1973). The level of raphanatin was essentially undiminished 23 days after the zeatin had been supplied. Raphanatin was also detected in Zea mays seedlings and roots and both raphanatin and zeatin 9-glucoside were identified as metabolites of zeatin in de-rooted lupin seedlings and poplar leaves (3.3.2. and 4.3.2.). By analogy with their stability in radish and corn, these metabolites are probably also stable, long lived metabolites in the latter two species. Although raphanatin appeared highly stable in de-rooted radish seedlings, a very slight conversion to zeatin was detected when the labelled metabolite was supplied to 3-day-old excised radish cotyledons (unpublished data). In

other studies not detailed herein the writer has obtained evidence that raphanatin is synthesised in developing radish seed (Raphanus sativus cv. long scarlet). Young seed pods (1.0 cm long) were injected with a [^{3}H]zeatin solution and left to mature on the plant. On attaining maturity, the seed was harvested and either extracted as dry seed or allowed to germinate on moist filter paper in the dark for about 30 hours prior to extraction. Raphanatin and zeatin 9-glucoside were identified as metabolites in both extracts by chromatographic methods. They accounted for approximately 10% of the radioactivity present in the seed. Data derived from a single experiment indicated that the ratio of raphanatin to zeatin 9-glucoside in the dry and germinating seed differed considerably, the values being 7:1 and 3:1, respectively. The above observations support the view that raphanatin and zeatin 9-glucoside may be storage forms of zeatin in radish seed.

The work of others in this laboratory has shown that exogenously supplied [^{3}H]BAP is rapidly metabolized by radish cotyledons and de-rooted seedlings (Wilson et al., 1974). The 7- and 9-glucosides of BAP have been identified as the two major metabolites together with the recently identified 3-glucoside as a minor, but highly active, metabolite (Letham et al., 1975; see appendix II). Both the 7- and 9-glucosides of BAP were virtually unmetabolized 30 hours after being supplied to de-rooted radish seedlings (via transpiration stream) and hence must be considered as very stable cytokinin metabolites

and potential storage forms. However, the 3-glucoside of BAP was quite unstable under similar conditions, being rapidly metabolized to BAP and to the 7- and 9-glucosides of BAP.

The O-glucosylzeatin and O-glucosyldihydrozeatin group of metabolites are other potential storage forms of cytokinins. It is possible that the presence of the glucose moiety on the side chain may protect the side chain from enzymic cleavage by cytokinin oxidase (Whitty and Hall, 1974; Paces et al., 1971). This oxidase activity, so far detected in tobacco and corn, was shown to be specific for the naturally occurring cytokinins isopentenyladenosine and zeatin riboside, degrading them to adenosine and adenine. A 'bound' form of cytokinin, purified from the roots of rice plants, has been tentatively identified as a zeatin glucoside (Yoshida and Oritani, 1972). The purified compound exhibited weak activity in the carrot callus growth assay, but a marked increase in activity was observed after acid hydrolysis or treatment with β-glucosidase. The active factor released by hydrolysis was chromatographically indistinguishable from zeatin. Studies in this laboratory have shown that the 7- and 9-glucosides of both zeatin and BAP are not degraded by β-glucosidase whereas the O-glucosyl metabolites of zeatin were susceptible to this enzyme. Therefore, it is very likely that the above bound cytokinin is O-glucosylzeatin.

Lupinic acid, the only metabolite reported herein possessing an intact zeatin moiety but lacking a conju-

gated sugar, could also be a storage form. It is only very weakly active in the radish bioassay, most of it remaining in the tissue unmetabolized while very minor conversion to zeatin was also detected.

On the basis of stability and ability to be slowly converted to free cytokinin, most of the cytokinin metabolites identified in this thesis could be considered as potential storage forms. The unequivocal chemical synthesis of all of them has now been achieved by colleagues in the Research School of Chemistry. Thus, the availability of these synthetic compounds will enable physiological studies to be conducted which will permit a better assessment of them as storage forms.

It is evident from the contents of this thesis that exogenously supplied cytokinins are converted to a diversity of metabolites with cytokinin activity. Thus, when zeatin riboside was supplied to poplar leaves, it was converted to four different metabolites all of which possessed an intact zeatin side chain which was O-glucosylated. All would be expected to exhibit cytokinin activity either *per se* or as a consequence of enzymic modification. Similarly, a highly active cytokinin such as zeatin could be active *per se* or a metabolite could be the active or functional form. The recognition of the active form or forms of cytokinins is essential for the elucidation of their mechanism of action. The identification of cytokinin metabolites is obviously of great significance in this regard. In this context it is relevant to note some

studies involving animal systems. For example, it has been established that the biologically significant form of vitamin D_3, which is involved *inter alia* in bone calcium mobilization, is not the vitamin itself but a hydroxylated form (Johnson *et al.*, 1975). Similarly, studies with the potent carcinogen aflatoxin B1 have indicated that a metabolite of this substance is the immediately active carcinogenic agent (Masri *et al.*, 1974).

At this point, it is appropriate to consider what basic criteria may be applied to assess the possibility that a compound is an 'active form' of a hormone. Firstly, the active form(s) should exhibit high activity in bioassays peculiar to the hormone. Secondly, the active form should be a short-lived (i.e. metabolically unstable) form of the hormone. Ready susceptibility to *in vivo* enzymic degradation is necessary to allow fine control of hormonally stimulated processes by 'switching off'. Thirdly, if a suspected active form of a hormone could be shown to stimulate a defined *in vitro* system (e.g. a protein or RNA synthesising system) then this could be taken as evidence for this form being the active form. In such a system, modification of the supplied hormone would be very unlikely. Fourthly, use could be made of 'transfer' experiments based on a bioassay system. In these a plant organ or tissue would be allowed to metabolize an exogenously supplied labelled hormone for a short time and then transferred to a hormone-free medium. In such an experiment, the levels of the various metabolites could be

monitored. If the level of a particular metabolite was found to decrease after transfer and this decrease was correlated with a falling off in the rate of the response being monitored (e.g. growth), then this metabolite could be an active form of the hormone.

None of the new metabolites reported in this thesis have been shown to fulfil all of the above criteria, but the 3-glucoside of BAP does satisfy the first two. In the radish cotyledon bioassay this metabolite is as active as BAP itself (see appendix I). When labelled 3-glucoside was supplied to 3-day-old excised radish cotyledons it was rapidly metabolized. Within 24 hours, 90% of the supplied radioactivity was detected in the 7- and 9-glucosides of BAP and some free BAP was also detected (Letham, unpublished data). These results strongly suggest that the 3-glucoside of BAP may be functionally important and work is in hand in this laboratory to further assess this possibility.

The retardation of senescence in attached and detached leaves and also leaf discs by exogenously supplied cytokinin is well documented and indicates an important role for cytokinins in the control of this complex physiological process. Proteolysis, RNA and chlorophyll degradation, all of which are associated with the process of leaf senescence, are known to be inhibited by cytokinins. The contribution which the results of this thesis make to our understanding of the regulation of leaf senescence is briefly discussed below.

Variations with leaf age in the composition of a naturally occurring cytokinin complex in poplar leaves have been reported (Hewett and Wareing, 1973b) and discussed previously (4.1.). These variations in cytokinin content could be a consequence of differences in the metabolism of cytokinins in senescing leaves compared with non-senescent leaves. Evidence to support this view was obtained in comparative studies of the metabolism of labelled zeatin riboside in senescing and non-senescing leaves of Populus nigra var. italica (see 4.3.4.). A marked difference in the major metabolites formed in the two sorts of leaves was detected. O-glucosylzeatin riboside and O-glucosyldihydrozeatin riboside were the major metabolites in green leaves whereas the free bases O-glucosylzeatin and O-glucosyldihydrozeatin were the major metabolites in yellow senescing leaves. These results clearly demonstrate differences in metabolism which could be of significance to an understanding of the process of senescence.

Lupinus angustifolius, the species of lupin used in the metabolism studies reported in chapter 3, is a species of plant in which the phenomenon of sequential senescence is readily observed. Under the glasshouse conditions used in this study, plants approximately 20 days old were beginning to exhibit fully senescent leaves at the stem base. Now that a range of zeatin metabolites in this species have been identified (chapter 3) it would appear to be the ideal plant in which to pursue studies concerning cytokinin involvement in sequential senescence and

abscission. Sequential senescence is thought to result from the inability of the older leaves (near the base) to compete effectively for nutrients exported from the root. Locally applied cytokinins are, however, known to be able to create a 'sink' effect at the site of application which results in delayed senescence in this region. In the intact plant, the older leaves may not receive adequate cytokinin from the root, or these leaves may convert cytokinin into weakly active or inactive forms (in lupin one of these might be lupinic acid). As a result the older lupin leaves may not compete effectively for essential nutrients. Based on the knowledge of cytokinin metabolites formed in lupins and reported in this thesis, it should now be possible to assess these possibilities by studies of cytokinin translocation and metabolism in mature plants.

The amino acid-zeatin conjugate (lupinic acid) was identified as a major metabolite in the leaves of derooted lupin seedlings. The functional significance of lupinic acid is as yet unknown, but the metabolite is of interest in connection with the antagonisms between kinetin and certain amino acids reported by Shibaoka and Thimann (1970). These workers found that the yellowing (senescence) of oat leaf segments in a cytokinin bioassay was promoted by the amino acid, L-serine, and to a lesser extent by L-cysteine and L-alanine. The L-serine (supplied at 30 mM) promotion of senescence counteracted the chlorophyll retention which was normally prolonged by kinetin (10^{-7} M) and other cytokinins. It was suggested

that serine acts, in some way, to promote proteolysis whereas kinetin acts not by actually stimulating general protein synthesis but by inhibiting protein degradation. At concentrations which promote chlorophyll retention, kinetin also inhibits the rise in ribonuclease levels in senescing oat leaves and this is completely reversed by simultaneously supplying serine; serine supplied alone to such leaves did not promote ribonuclease levels above those of control leaves. These authors propose that even though serine does not promote ribonuclease levels, the fact thatit reverses kinetin inhibition of the rise in ribonuclease suggests it may have the same action in untreated leaves by way of endogenous cytokinins. In the light of the purification and identification of lupinic acid, an alternative explanation of these results can be suggested. Serine as its O-acetyl derivative, is very probably the donor of the alanine moiety in lupinic acid (see 5.3.). Hence serine, when supplied in very high and unphysiological concentrations, probably combines with all free kinetin to form the amino acid-kinetin analogue of lupinic acid which would be relatively inactive. This would allow ribonuclease levels to rise, proteolysis to increase and other symptoms of senescence to become obvious; all the effects of serine may be interpreted as being the results of its inactivation of the cytokinin.

The ability of cytokinins to delay senescence is the basis for several cytokinin bioassays. In particular, species of the Gramineae (e.g. barley) have found favour

in this regard (see 2.1.). These assays are commonly used because of the short period between setting up the assay and obtaining the result. However, these assays generally suffer from one drawback; in contrast to their relative activities in growth assays, synthetic cytokinins such as BAP and kinetin exhibit markedly greater activity than the naturally occurring cytokinin zeatin (Letham, 1967a; Fawcett and Wright, 1968; Varga and Bruinsma, 1973). This has been observed with oat, wheat and Zea mays leaves. Comparative studies of zeatin and BAP metabolism in a graminaceous species (Zea mays) are reported in this thesis. BAP was found to be much less susceptible to metabolism than zeatin. It formed only one major metabolite, the 9-glucoside, whereas zeatin was more extensively metabolized, a considerable portion being converted to adenine and adenosine. Probably this observed stability of BAP compared with the natural hormone is the basis for its greater activity in the senescence assays. Zeatin could well be as active if it was able to persist in the tissue, but by being susceptible to the available degradative enzymes, it is rapidly removed and senescence allowed to occur.

The studies of cytokinin metabolism reported in this thesis have resulted in the identification of a number of new cytokinin metabolites including some of unusual structure. Methods have been developed which enable the separation and chromatographic characterization of the

metabolites. The chemical synthesis of these new metabolites by co-workers has provided material for future physiological studies. While the results presented have made some direct contribution to our knowledge of storage forms of cytokinins and possibly to regulation of leaf senescence, the most significant feature of the work reported is the chemical basis which is provided for a variety of worthwhile physiological studies.

APPENDIX

Reprinted from 61

Biochimica et Biophysica Acta, 399 (1975) 61—70

BBA 27687

REGULATORS OF CELL DIVISION IN PLANT TISSUES

XXIII. THE IDENTITY OF AN UNUSUAL METABOLITE OF 6-BENZYLAMINOPURINE*

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(Received January 27th, 1975)

Summary

When the cytokinin 6-benzylaminopurine was supplied to de-rooted radish seedlings, the principal metabolites formed were the 7- and 9-glucosides. However the cytokinin activity of these glucosides was much less than that of a minor metabolite. This metabolite was purified (yield 550 μg from 40 600 seedlings), identified as 6-benzylamino-3-β-D-glucopyranosylpurine and synthesized. It is the first compound with a glycosidic linkage at position 3 of a purine ring to be isolated from a plant tissue.

Introduction

The phytohormone zeatin (6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine), a cytokinin, is now known to be rapidly metabolized in plant tissues. The principal metabolites identified are: 7-glucosylzeatin, 9-glucosylzeatin, zeatin riboside, zeatin riboside 5′-monophosphate, and dihydrozeatin (6-(4-hydroxy-3-methylbutylamino)purine) and its riboside and riboside 5′-phosphate [1—6]. The synthetic cytokinin, 6-benzylaminopurine, is also converted to 7- and 9 glucosides and to the riboside and riboside 5′-monophosphate [2,7—10]. When 6-benzylaminopurine is supplied to de-rooted radish seedlings, the principal metabolites are the 7- and 9-glucosides [2,8]. However a minor metabolite exhibits cytokinin activity markedly greater than that of these glucosides. Because of this high activity, the metabolite is of considerable physiological interest; its isolation and identification are detailed herein.

* Part XXII: Gordon, M.E. and Letham, D.S. (1975) Aust. J. Plant Physiol., in press.

Experimental

Preparation of 6-[^{3}H] benzylaminopurine

6-[G-^{3}H] Benzylaminopurine (25 Ci/mol) was prepared by stirring 6-benzylaminopurine with tritiated water and a platinum catalyst under reflux. The crude product was freed from exchangeable ^{3}H and then purified by thin-layer chromatography, by precipitation from an acid solution as a result of pH adjustment to 7.0, and finally by crystallization from ethanol/water.

Uptake and extraction methods

The roots were excised from 9-day-old radish (*Raphanus sativus* L. cv. Long Scarlet) seedlings and the cut ends of the hypocotyls were placed in 6-benzylaminopurine solution (for times and concentrations, see below). During uptake of the cytokinin, the de-rooted seedlings were placed in a gentle air current at 23° C under continuous weak fluorescent light (700 lux). For extraction, the seedlings were dropped into 80% methanol or ethanol (8 ml per g of tissue) at 65° C, maintained at this temperature for 4—5 min and then cooled rapidly. Homogenization and filtration yielded an extract which was evaporated under vacuum.

Chromatographic and electrophoretic methods

Silica gel for both analytical and preparative thin-layer chromatography was Merck PF_{254}; for preparation of borate-impregnated layers, the silica gel was mixed with 0.05 M $Na_2B_4O_7$ for spreading. Layers of DEAE-cellulose with a fluorescent indicator [3] were sprayed with 0.05 M $Na_2B_4O_7$ and allowed to dry prior to use. Cellulose phosphate paper (Whatman P81) was equilibrated to pH 4.9 by washing exhaustively with 1 M ammonium phosphate (pH 4.9) and then with water. Paper electrophoresis was performed on Whatman 3 MM paper (25 V/cm, 2.5 h) held between metal plates maintained at 15° C.

Ultraviolet-absorbing zones on chromatograms were located with a short-wave ultraviolet lamp. Zones from preparative thin-layer cchromatograms were packed into a column and eluted with methanol/water/acetic acid (80 : 20 : 1, by vol.). Eluents used to elute zones for determination of radioactivity were: 0.2 M acetic acid (silica gel layers), 0.05 M Na_2HPO_4 (cellulose phosphate paper), and water (paper electrophoretograms). Determination of radioactivity in a liquid scintillation spectrometer and autoradiography of thin-layer chromatograms were performed as detailed previously [3].

The following chromatography solvents (proportions by vol.) were used: A, *n*-butanol/acetic acid/water (12 : 3 : 5); B, *n*-butanol/14 M NH_4OH/water (6 : 1 : 2, upper phase); C, acetone/water (4 : 1); D, *n*-butanol saturated with water; E, ethyl methyl ketone/acetic acid/water (16 : 1 : 4); F, chloroform/methanol (24 : 1); G, ethanol/water (2 : 1) saturated with $Na_2B_4O_7$; H, 2-ethoxyethanol/water (3 : 1) saturated with $Na_2B_4O_7$.

Determination of mass spectra

Spectra of underivatized compounds were recorded by solid sample probe introduction into either an AEI MS-902 mass spectrometer or a Varian CH7 instrument operated at 70 eV. Trimethylsilyl (Me_3Si) derivatives were prepared

by reaction in pyridine with Regisil RC-2 (*N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane) and their spectra obtained by combined gas chromatography-mass spectrometry on a Varian MAT 111 system. Conditions used were: injector 280°C, glass column packed with 2% OV-17 on 80—100 Gas Chrom Q programmed from 240 to 300°C, separator and line 300°C.

Isolation of compound 19

De-rooted radish seedlings (40 600, total weight 4.1 kg) were supplied with 6-benzylaminopurine (0.12 mM) for 24 h and extracted with 80% ethanol (see above). An aqueous solution (2.5 l) of the water-soluble fraction of the evaporated extract was shaken with four 2.5-l volumes of water-saturated *n*-butanol. The residue obtained by evaporation of the extract in vacuo was dissolved in water and the solution (900 ml, pH adjusted to 3.0) was percolated through a column of Whatman P1 cellulose phosphate powder (420 g, NH_4^+ form equilibrated to pH 3.1) which was washed with 0.05 M acetic acid (3 l) followed by water (6 l). The eluate obtained by elution of the column with 0.45 M NH_4OH (10 l) was evaporated and the residue suspended in 50% ethanol (50 ml). The suspension was centrifuged and the supernatant applied to 30 20 cm × 20 cm × 0.1 cm layers of silica gel which were developed with solvent A. The zones containing compound 19 (R_F 0.31) were eluted and rechromatographed on 20 of the above silica gel layers using solvent B, each plate being developed three times. Compound 19 was evident as a very faint ultraviolet absorbing zone (R_F 0.29) which travelled just in front of the intense zone of 6-benzylamino-7-glucosylpurine (R_F 0.27). The zones containing compound 19 were eluted and further purified by thin-layer chromatography on silica gel using solvent C. The eluate of the desired zone (R_F = 0.71) was evaporated and the residue dissolved in water (6 ml). The solution was shaken with three 6-ml volumes of water-saturated *n*-butanol and the extracted fraction was chromatographed on Schleicher and Schull 2040b paper (washed exhaustively with 20% ethanol) with solvent D (atmosphere saturated with NH_3). The zone at R_F 0.54 was eluted with ethanol and evaporated yielding chromatographically homogeneous compound 19, 550 μg. A portion of the product was crystallized from ethanol/petroleum yielding crystalline compound 19, micro melting point (Kofler apparatus) 252—253°C. The mass spectrum is presented in Fig. 1. Mass spectrum of Me_3Si derivative (principal *m/e* values > 120 with relative intensities in parentheses): 675 (M^+, 0.1), 660 (0.3), 450 (6), 360 (7), 298 (7), 271 (8), 257 (16), 254 (6), 232 (11), 224 (16), 218 (26), 217 (60), 204 (5), 191 (7), 169 (10), 149 (7), 147 (31), 129 (11); base peak 73 (100).

Acid hydrolysis of compound 19

Compound 19 (40 μg) and 0.6 M trifluoroacetic acid (1 ml) were heated at 95°C for 3 h. The solution was then evaporated in vacuo and the residue subjected to thin-layer chromatography on silica gel (solvent B) to detect 6-benzylaminopurine. The metabolite was also hydrolyzed by a sulphonic acid resin. Compound 19 (10 μg) was dissolved in 0.1 M acetic acid (0.10 ml) and the solution was stirred with the cation-exchange resin (Zeo-Karb 225, Permutit SRC 14, H^+ form, 10 mg) for 15 min. The suspension was then heated at

120° C (autoclave) for 1 h and finally centrifuged. The supernatant was evaporated on a silica gel thin-layer and chromatographed with solvent E. The developed chromatogram was dried, placed in an atmosphere containing NH_3 to neutralize acetic acid, redried, and finally sprayed with a solution containing glucose oxidase, peroxidase and 3,3′-dimethoxybenzidine to detect glucose [11]. The procedure readily detected 1 μg of glucose after thin-layer chromatography.

Hydrolysis of compound 19 with glucosidase

Compound 19 (about 12 μg) was dissolved in acetate buffer (0.03 M, pH 5.3, 60 μl) containing almond β-glucosidase (EC 3.2.1.21, source Sigma Chemical Co., 0.5 mg/ml) and the solution incubated at 35° C for 4 h. The hydrolyzate was evaporated onto a silica gel layer and chromatographed (solvent E) to detect 6-benzylaminopurine.

Synthesis of 6-benzylamino-3-β-D-glucopyranosylpurine

6-Benzylaminopurine (2 mmol) was reacted with α-D-tetra-*O*-acetylglucopyranosyl bromide (2.4 mmol) in *N*,*N*-dimethylformamide (5 ml) at 100° C for 20 h and an aliquot of the very dark product was fractionated by preparative thin-layer chromatography on silica gel (solvent F). The principal band, which was just above a weak zone of unreacted 6-benzylaminopurine, was eluted with chloroform/methanol (24 : 1, v/v) and the evaporated eluate (300 mg) crystallized from methanol to yield 3-β-D-tetra-*O*-acetylglucopyranosyl-6-benzylaminopurine, melting point 205—206° C (uncorrected). The ultraviolet spectra were almost identical to those of compound 19. NMR spectrum (100 MHz, C^2HCl_3): δ 8.33 and 7.67 (both singlet and 1H; purine ring H), 7.28 (singlet, 5H, C_6H_5-), 6.30 (doublet, J = 8.6 Hz, 1H, H_1'), 5.65—5.15 (multiplet, 3H.; H_2', H_3', H_4'), 4.94 (doublet of doublet, J = 5.5 and 15 Hz, 2H, benzyl methylene), 4.45—3.90 (multiplet, 3H, H_5' and H_6'), 2.1—2.0 (multiplet, 9H, acetyl), and 1.68 ppm (singlet, 3H, acetyl). Mass spectrum (principal *m/e* values and relative intensities): 555 (M^+, 32), 331 (6), 226 (22), 225 (83), 224 (40), 169 (62), 145 (6), 139 (8), 127 (20), 120 (14), 115 (7), 109 (55), 106 (41), 103 (6), 97 (11), 91 (53), 81 (9), 65 (10), 60 (8) and 43 (100).

The chromatogram zone (176 mg) above that of the foregoing 3-glucoside tetraacetate was the isomeric 9-β-D-tetra-*O*-acetylglucopyranosyl-6-benzylaminopurine, melting point 156—157° C (uncorrected); λ_{max} in MeOH (neutral, acidic and basic): 265—270 nm. The mass spectrum was almost identical to that of the 3-glucoside tetraacetate. Treatment with methanolic NH_3 for 2 h at room temperature yielded 6-benzylamino-9-β-D-glucopyranosylpurine, identical to an authentic sample [2].

Treatment of the above 3-glucoside tetraacetate with methanolic NH_3 for 2 h at room temperature yielded 6-benzylamino-3-β-D-glucopyranosylpurine, melting point 248—249° C (uncorrected); ultraviolet spectra identical to those of compound 19. Mass spectrum (*m/e* values > 90 and relative intensities): 387 (M^+, 1.3), 268 (0.6), 267 (0.3), 266 (0.2), 254 (3.2), 226 (14), 225 (93), 224 (38), 209 (3), 197 (3), 148 (10), 121 (8), 120 (19), 119 (12), 106 (100), 104 (4), 93 (17), 91 (67). The compound was very slowly hydrolyzed by β- but not α-glucosidase.

Detection of compound 19 by mild procedures

Seedlings supplied with 6-[^{3}H]benzylaminopurine were extracted at 4°C. The extract was evaporated at 25° C and an aqueous solution of the residue was shaken with *n*-butanol. The evaporated (25°C) extract was subjected to chromatography on cellulose phosphate paper (NH_4^+ form) equilibrated to pH 4.9 (solvent water) and to paper electrophoresis (0.05 M acetate, pH 4.7). In these procedures, advantage was taken of the difference in basic pK_a between the 3-glucoside of 6-benzylaminopurine (pK_a determined spectrophotometrically at 20°C, 5.50) and the 7- and 9-glucosides (probable pK_a values approx. 3.2; see reported pK_a values for 7- and 9-substituted adenines [12]). This difference enabled the 3-glucoside to be readily separated from the 7- and 9-glucosides by the methods mentioned.

Results

The metabolite with high cytokinin activity was first detected when 6-[^{3}H]benzylaminopurine (60 μM) was supplied for 8 h to de-rooted radish seedlings which were then transferred to water for 16 h. When the extract, purified by butanol extraction and elution from cellulose phosphate (method similar to that used in the isolation of compound 19), was subjected to two-dimensional thin-layer chromatography on silica gel, the metabolite was detectable by autoradiography and under ultraviolet light and is designated compound 19 in Fig. 2. In this figure, compounds revealed by ultraviolet light are numbered 1—19 and those identified are as follows: 3, 6-benzylaminopurine; 4, 6-benzylamino-9-glucopyranosylpurine; 5, adenosine; 9, 6-benzylamino-7-glucosylpurine; 20, 6-benzylamino-9-β-D-ribofuranosylpurine. To separate compound 19 (very faint spot) completely from compound 9 (intense spot), the chromatogram was developed at least twice in the second dimension. Compounds 3, 4, 5, 9, 10, 19 and 20 were labelled, and the relative cpm in these substances were (radioactivity in compound 9 being taken as 100) 11, 41, 0.2, 100, 3, 7 and 3, respectively. Although compound 19 was a very minor metab-

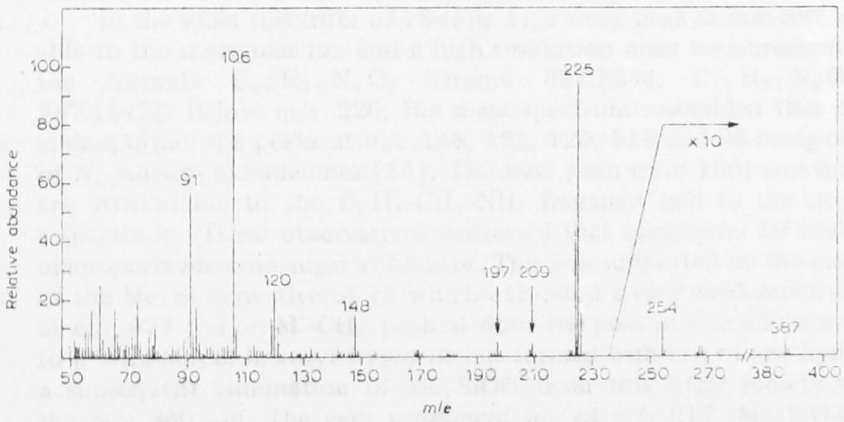

Fig. 1. The mass spectrum of compound 19 (mass spectrometer AEI MS-902, 70 eV; source temperature approx. 270° C).

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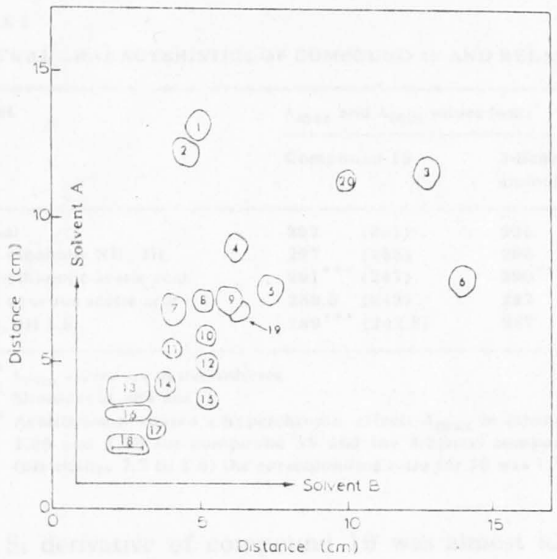

Fig. 2. A two-dimensional silica gel thin-layer chromatogram of the fraction of radish seedling extract eluted from a cellulose phosphate column. The chromatogram was developed first with solvent A and then with solvent B. The origin is denoted by the barred line and is enclosed by spot 18; the solvent travelled 15 cm from the origin in each direction.

olite, the eluate of 19 induced a growth increment in the radish cotyledon cytokinin bioassay [13] which was greater than that evoked by the eluates of compounds 4 and 9 (the 9- and 7-glucosides). To isolate 19 in amounts adequate for chemical characterization, 40 600 seedlings supplied with 6-benzylaminopurine were extracted. The purified metabolite exhibited the following R_A values (R_F of 19/R_F of adenosine) on thin layers of silica gel: 0.97, 0.77 and 0.73 with solvents A, B, and E, respectively; on cellulose layers the R_A values were 2.63 and 1.33 with solvents B and E, respectively.

In the mass spectrum of 19 (Fig. 1), a weak peak at *m/e* 387 was attributable to the molecular ion and a high resolution mass measurement established the formula $C_{18}H_{21}N_5O_5$ (found 387.1544; $C_{18}H_{21}N_5O_5$ requires 387.1542). Below *m/e* 226, the mass spectrum resembled that of 6-benzylaminopurine, the peaks at *m/e* 148, 121, 120, 119 and 93 being characteristic of N_6-substituted adenines [14]. The base peak (*m/e* 106) and that at *m/e* 91 are attributable to the C_6H_5-CH_2-NH- fragment and to the tropylium ion, respectively. These observations suggested that compound 19 was a 6-benzylaminopurine-hexose sugar conjugate. This was supported by the mass spectrum of the Me_3Si derivative of 19 which exhibited a very weak molecular ion peak at *m/e* 675 and an M^+-CH_3 peak at 660. The peak at *m/e* 450 was attributable to a tetra-Me_3Si hexosyl fragment ion formed with loss of one hydrogen while a subsequent elimination of Me_3SiOH from this sugar moiety would yield the *m/e* 360 ion. The very prominent ion at *m/e* 217 (Me_3SiO-CH=CH-CH-$OSiMe_3$) and ions of *m/e* 129, 147, 191, 204, 243 and 305 in the spectrum are characteristic of carbohydrate Me_3Si derivatives [15]. The spectrum of the

TABLE I

SPECTRAL CHARACTERISTICS OF COMPOUND 19 AND RELATED SUBSTANCES

Solvent	λ_{max} and λ_{min} values (nm)*		
	Compound 19	3-Benzyl-6-benzyl-aminopurine	Acid hydrolysis product of 19
Ethanol	297 (251)	296 (252)	270 (232)
0.4 M ethanolic NH_4OH	297 (252)	296 (252)	275** (241)
0.2 M ethanolic acetic acid	291*** (247)	290*** (248)	
0.1 M aqueous acetic acid	288.5 (242)	287 (243)	274 (234)
Water, pH 1.6	289*** (242.5)	287 (243)	

* λ_{min} values are in parentheses.

** Shoulder at 284 nm.

*** Acidification caused a hyperchromic effect; A_{max} in ethanol plus acetic acid/A_{max} in ethanol = 1.25 and 1.30 for compound 19 and the 3-benzyl compound, respectively. In aqueous solution (pH change 7.7 to 1.6) the corresponding ratio for 19 was 1.37.

Me_3Si derivative of compound 19 was almost identical to that of the Me_3Si derivative of 6-benzylamino-9-β-D-glucopyranosylpurine.

Hydrolysis of compound 19 with acid yielded a compound which exhibited spectral characteristics (Table I) identical to those of 6-benzylaminopurine and co-chromatographed with 6-benzylaminopurine during thin-layer chromatography on cellulose, silica gel and polyamide. Hydrolysis with a polystyrene sulphonic acid resin yielded a sugar identified as glucose using glucose oxidase. The glycosidic linkage of compound 19 was cleaved by β-glucosidase, but not by α-glucosidase; however, the rate of hydrolysis was very slow relative to that of salicin. Under the hydrolysis conditions used, salicin was completely hydrolyzed to *o*-hydroxybenzyl alcohol, but only about 30% of compound 19 was degraded to 6-benzylaminopurine.

The ultraviolet spectra of 19 resembled those recorded for N_6,3-disubstituted adenines [16] and when the spectra for 19 and 3-benzyl-6-benzylaminopurine were determined in the same solvents, the spectral characteristics were almost identical (Table I). The above observations indicated that compound 19 was 6-benzylamino-3-β-glucosylpurine. During paper electrophoresis (0.026 M borate, pH 9.2), 19 exhibited a very low mobility equal to that of 6-benzylamino-9-β-D-glucopyranosylpurine and markedly less than that of 6-benzylamino-9-β-D-glucofuranosylpurine (mobility of compound 19/mobility of the 9-glucofuranoside = 0.12). Hence compound 19 appeared to be a glucopyranoside.

To synthesize 6-benzylamino-3-β-D-glucopyranosylpurine, 6-benzylaminopurine was reacted with α-D-tetra-*O*-acetylglucopyranosyl bromide and the major product deacetylated in methanolic NH_3. When 3-β-D-tetra-*O*-acetylglucopyranosyl-6-benzylaminopurine, the intermediate in the synthesis of the 3-glucoside, was heated to 250°C for 20 min, a 30% conversion to 9-β-D-tetra-*O*-acetylglucopyranosyl-6-benzylaminopurine occurred. The synthetic 3-glucoside and compound 19 could not be distinguished by mass and ultraviolet spectra or by the mass spectra of their Me_3Si derivatives. They exhibited identical cytokinin activity in the radish cotyledon cytokinin bioassay [13]. The

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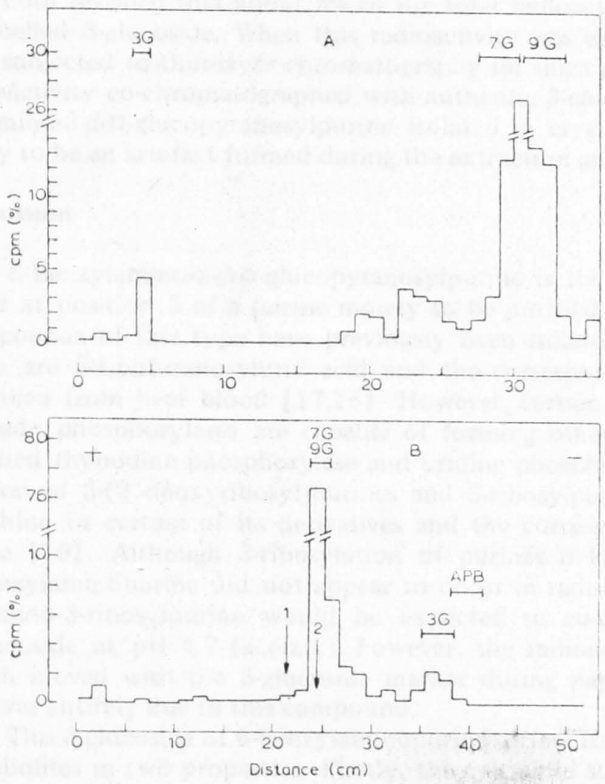

Fig. 3. The distribution of radioactivity over a cellulose phosphate paper chromatogram (A) and a paper electrophoretogram (B) of extract of radish seedings prepared at 4°C. In A, the solvent was water; in B, the buffer was 0.05 M acetate pH 4.7. Marker compounds (location indicated by barred lines) are the 3-, 7- and 9-glucopyranosides of 6-benzylaminopurine (3G, 7G and 9G, respectively) and 9-(3-aminopropyl)-6-benzylaminopurine (APB). In B, arrow 1 denotes the origin and arrow 2 indicates the location of an indicator of electroendosmotic flow (6-chloropurine riboside). Radioactivity in each zone is expressed as a percentage of the total.

two compounds could not be separated by thin-layer chromatography on cellulose, silica gel, polyamide, borate-impregnated silica gel (solvents G and H), or borate-impregnated DEAE-cellulose (solvent H). The borate systems readily separate glucopyranosides from glucofuranosides because of complex formation between borate and the unconstrained 5',6'-diol grouping in the furanosides. Furthermore the synthetic 3-glucoside and compound 19 were indistinguishable by paper electrophoresis (phosphate pH 4.7, borate pH 9.2) and exhibited the same melting point, unaltered by admixture. The above evidence established that compound 19 was 6-benzylamino-3-β-D-glucopyranosylpurine.

To eliminate the possibility that the 3-glucoside metabolite isolated could have been an artefact formed by the extraction and fractionation procedures, a milder extraction of seedlings supplied with 6-[^{3}H]benzylaminopurine was performed and fractionation procedures used which did not involve acidic or basic conditions. Chromatography of the butanol fraction of the extract (see Experimental) on cellulose phosphate paper (Fig. 3A) and paper electrophoresis (Fig.

3B) both revealed that about 5% of the total radioactivity moved with added unlabelled 3-glucoside. When this radioactivity was eluted from both systems and subjected to thin-layer chromatography on silica gel and cellulose, all the radioactivity co-chromatographed with authentic 3-glucoside. Hence the 6-benzylamino-3-β-D-glucopyranosylpurine isolated in crystalline form is most unlikely to be an artefact formed during the extraction and fractionation.

Discussion

6-Benzylamino-3-β-D-glucopyranosylpurine is the first compound with a sugar at position 3 of a purine moiety to be purified from a plant. Only two compounds of this type have previously been isolated from natural sources; these are 3-ribofuranosyluric acid and the corresponding 5'-phosphate both obtained from beef blood [17,18]. However, certain purified pyrimidine nucleoside phosphorylases are capable of forming other 3-ribosylpurines. Thus purified thymidine phosphorylase and uridine phosphorylase catalyze the formation of 3-(2'-deoxyribosyl)purines and 3-ribosylpurines, respectively, from xanthine or certain of its derivatives and the corresponding pentose 1-phosphate [19]. Although 3-ribosylation of purines is known, 3-ribosylation of 6-benzylaminopurine did not appear to occur in radish seedlings. Thus 6-benzylamino-3-ribosylpurine would be expected to co-electrophorese with the 3-glucoside at pH 4.7 (acetate); however, the radioactivity in radish extract which moved with the 3-glucoside marker during paper electrophoresis (Fig. 3B) was entirely due to this compound.

The 3-glucoside of 6-benzylaminopurine differs from the 7- and 9-glucosyl metabolites in two properties. Firstly, the cytokinin activity of the 3-glucoside markedly exceeded that of the 7- and 9-glucosides. In the radish cotyledon assay, the lowest detectable concentration of the 7- and 9-glucosyl metabolites was about 1 μM, while the 3-glucoside was still active at 0.02 μM. At this concentration it was slightly more active than 6-benzylaminopurine, but the two compounds induced identical growth increments over the concentration range 0.1—4 μM. Secondly, the 3-glucoside was hydrolyzed slowly by almond β-glucosidase whereas the 7- and 9-glucosyl metabolites were not hydrolyzed at a detectable rate by either α- or β-glucosidase.

Tobacco callus tissue has been cultured in the presence of labelled 6-benzylaminopurine and a paper chromatogram of the tissue extracts was assayed for cytokinin activity [7]. The activity associated with the zone containing the 7-glucoside was attributed entirely to this compound. Because the 3-glucoside is markedly more active than the 7-glucoside, and since these compounds exhibit very similar chromatographic properties on cellulose, the above conclusion [7] requires reassessment.

The mass spectrum of the Me_3Si derivative of 6-benzylamino-3-β-D-glucopyranosylpurine was virtually identical to that of the Me_3Si derivative of 6-benzylamino-9-β-D-glucopyranosylpurine (spectra obtained by combined gas chromatography-mass spectrometry). This appears to be a consequence of thermal rearrangement of the 3-glucoside derivative to the 9-glucoside derivative. An analogous thermal rearrangement involving the corresponding tetraacetates was mentioned in Results. These are the first reported rearrangements of 3-substi-

tuted N_6-alkyladenines to 9-substituted compounds. The presence of an N_6-acyl group was previously regarded as essential for the transfer of a substituent from the 3 to the 9 position [20]. For example the rearrangement of 3-alkyl and 3-ribosyl derivatives of 6-benzamidopurine to 9-substituted derivatives has been reported previously [21—23].

Some evidence suggests that the 7-glucosides of cytokinins are stable metabolites and may be storage forms [3], but there is no information at present concerning the stability and physiological significance of the 3-glucoside of 6-benzylaminopurine. An investigation to provide such information would involve assessment of the level of labelled 3-glucoside in plant tissue and this could be readily achieved by methods described herein, namely chromatography on cellulose phosphate paper (Fig. 3A) and paper electrophoresis (Fig. 3B). Because of the high cytokinin activity of the 3-glucoside, it is a metabolite which merits further physiological study.

Acknowledgement

Thanks are due to Dr D.D. Perrin, Department of Medical Chemistry, John Curtin School of Medical Research, Australian National University for determination of the pK_a value.

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